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ABSTRACT

Characterization of the Murine Salmonella typhimurium-Specific Primary B-Cell Repertoire

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Doctor of Philosophy, 1984

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Recent studies suggest that B cells play an important role in the immune response to Salmonella typhimurium which causes a typhoid-like disease in mice. One approach to understanding the role of B cells in the immune response to S. typhimurium would be to analyze the repertoire of salmonella-specific B cells in normal and salmonella-susceptible mice. However, to date no studies of the B-cell repertoire specific for salmonella, nor any other bacterial infectious disease agent, have yet been undertaken. The splenic focus system permits the identification and characterization of individual B cells and their antibody products and an estimate of the size of the antigen-specific repertoire. Therefore, the splenic focus system has been modified to analyze the B-cell repertoire specific for S. typhimurium strain TML (TML). The studies described herein define the conditions for obtaining monoclonal responses to acetone-killed and dried

TML (AKD-TML) in salmonella-resistant CBA/Ca mice and salmonella-susceptible CBA/N mice.

The frequency of primary TML-specific splenic B cells in CBA/Ca mice is approximately 1 per 1 x 10^5 B cells. In contrast, the frequency of memory TML-specific cells is approximately 1 per 5-8 x 10^3 splenic B cells and the majority of these B cells are specific for LPS. These results suggest that the frequency of primary TML-specific B cells is extremely low and that it expands 15- to 20-fold after antigen exposure. Interestingly, less than 30% of the primary B cells are specific for the LPS molecule which is considered to be the major antigenic determinant on Salmonella organisms. Furthermore, the majority of the LPS-specific anti-TML antibody-producing clones are directed against the LPS O-antigen region. Conversely, less than half of the memory LPS-specific anti-TML B-cell clones are directed against the O-antigen. The remaining half are directed against the KDO or lipid A region of the LPS molecule. These results indicate that after contact with antigen there is preferential expansion of LPS-specific B-cell clones.

Unlike B-cell responses to chemically-defined antigens, TML stimulates very little IgG1 antibody. IgG2 and IgA isotypes appear to play an important role in anti-TML antibody responses, although all heavy chain classes are produced to some extent. Salmonella-susceptible, antibody defective CBA/N mice, on the other hand, express

no primary or secondary TML-specific precursors after stimulation with AKD-TML. However, after three immunizations, the CBA/N tertiary frequency is similar to that of primary CBA/Ca TML-specific B cells. Normal mice have two major B-cell subpopulations, Lyb-5^+ and Lyb-5^-. Since CBA/N mice only express the splenic Lyb-5^- B-cell subset, it appears that, like the PC-specific response in these mice, Lyb-5^- TML-specific B cells may need to differentiate into memory cells before they can mature into antibody-secreting cells. Furthermore, although the majority of tertiary CBA/N TML-specific clones are directed against the LPS molecule, as are memory CBA/Ca clones, most of these B cells are specific for the O-antigen region. Thus, CBA/N mice appear to lack the KDO/lipid A-specific B-cell subset.

The isotype profile and fine specificity analysis of TML-specific clones reflect differences among all three B-cell subsets (primary CBA/Ca, memory CBA/Ca, and tertiary CBA/N B cells). The results also indicate an abnormal distribution of S. typhimurium B-cell clones in CBA/N mice which may account for their susceptibility to S. typhimurium.

CHARACTERIZATION OF THE MURINE *SALMONELLA TYPHIMURIUM*-SPECIFIC B-CELL REPERTOIRE

by

Lise Weisman Duran

Dissertation submitted to the
Faculty of the Department of Microbiology
Graduate Program of the Uniformed Services Unversity of
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requirements for the degree of
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Dedication

First, to my husband, Stan, who has been my strength and driving force. This dissertation is as much a credit to his efforts as it is to mine, for without his constant love, companionship, and solid shoulder to lean upon, the completion of this thesis would not have been possible.

And, to my parents, Herman and Margaret Weisman, who have given me a lifetime of love, encouragement, and support. They have always shown complete faith in my ability to achieve in every endeavor I have chosen to pursue.

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I. Introduction

Salmonella typhimurium is a gram-negative, facultative, intracellular bacterium which multiplies in the phagocytic cells of the reticuloendothelial system. Infection with this organism causes a typhoid-fever like disease in mice. Most strains of mice challenged with as many as 1×10^4 *S. typhimurium* are able to restrict the growth of this organism and, as a consequence, are able to survive the infection. In contrast, other strains of mice succumb to infection with as few as 1-10 bacteria (Robson and Vas, 1972; Plant and Glynn, 1976; Hormaeche, 1979b). This differential response among various inbred strains of mice to *S. typhimurium* is controlled by at least three distinct host genes: *Ity*, *Lps*, and *xid*. The *Ity* gene is located on chromosome 1 (Plant and Glynn, 1979; O'Brien *et al.*, 1980a) and exists in two allelic forms: the dominant, resistance (Ity^r) and the recessive, susceptibility (Ity^s) alleles. Mice which are homozygous for the Ity^s allele are unable to control the rapid net multiplication of *S. typhimurium* in the tissues of their reticuloendothelial system (RES) and they die early after infection. Conversely, mice which carry the Ity^r allele are able to curb net multiplication and they survive the infection (Robson and Vas, 1972; Plant and Glynn, 1976; Hormaeche, 1979a; O'Brien *et al.*, 1980b). In comparison, the *Lps* gene is located on chromosome 4 (Watson *et al.*, 1978). Mice

that carry the normal allele (*Lps*^{n}) are reactive to low doses of lipopolysaccharide (LPS) whereas strains of mice that are homozygous for the defective mutant allele (*Lps*^{d}) are hyporesponsive to all doses of LPS (reviewed in Morrison and Ryan, 1979) and are highly susceptible to *S. typhimurium* (Robson and Vas, 1972; O'Brien *et al.*, 1979).

Mice which express the *Ity*^{s} salmonella-sensitive phenotype are very similar to those mice which express the *Lps*^{d} phenotype. Both *Ity*^{s} and *Lps*^{d} mice, in contrast to *Ity*^{r} and *Lps*^{n} mice, are unable to control the initial net multiplication of salmonellae in their spleens. As a result, these mice, which express both of these recessive alleles homozygously, generally die early (<10 days) after infection, and, regardless of the route of inoculation, the median lethal dose ($L.D._{50}$) of *S. typhimurium* for these mice is <20 (Robson and Vas, 1972; Plant and Glynn, 1976; Hormaeche, 1979a, O'Brien *et al.*, 1980b). Neither *Ity*^{s} nor *Lps*^{d} mice can be protected by passive transfer of immune serum (see O'Brien *et al.*, 1982b). However, both *Lps*^{d} and *Ity*^{s} mice can be converted to the salmonella-resistant phenotype by reconstitution with *Lps*^{n} or *Ity*^{r} bone marrow cells, respectively (Hormaeche, 1979c; O'Brien *et al.*, 1981; O'Brien *et al.*, 1982a). These findings support the hypothesis that a bone marrow-derived, radioresistant cell is the effector cell responsible for the phenotypic expression of both *Ity* and *Lps* genes. More direct support for this theory was provided by the studies of O'Brien *et*

al. (1979a). These investigators showed that silica treatment of innately resistant mice caused: 1) a rapid increase in the number of bacteria found per spleen 1 to 3 days post-infection; and 2) an early death (<10 days) with a marked decrease in the $L.D._{50}$ when compared to non-silica-treated mice. Since previous reports showed that silica selectively reduced macrophage function (Kessel et al., 1963; O'Brien et al., 1979a), these studies suggested that the increased salmonella-susceptibility of innately resistant mice early in the infection was due to macrophages and was consistent with the hypothesis that the macrophage was the most important effector cell in the expression of Ity or Lps.

The mechanism(s) by which the products of these two genes control the early response of mice to S. typhimurium infection is not clear. In vivo studies which utilized Ity^s and Ity^r mice showed that the rate of clearance of S. typhimurium from the bloodstream and the uptake of the microbe by splenic and hepatic macrophages was not different for resistant and susceptible mice (Gröschel et al., 1970; Swanson and O'Brien, 1983). Therefore, the product(s) of the Ity gene does not appear to regulate phagocytosis of bacteria by RES macrophages. Swanson and O'Brien (1983) extended these initial observations by the demonstration that the bacterial counts in the spleens of Ity^s mice reached significantly greater levels than the

counts obtained from *Ity*^{r} spleens by 24 hours post-infection.

The findings of these investigators were confirmed *in vitro* using splenic and peritoneal macrophage cultures (Maier and Oels, 1972; Blumenstock and Jann, 1981; Lissner *et al.*, 1983). Previous studies had shown that lysosomal enzymes and oxygen-dependent bactericidal events are involved in early membrane-associated bactericidal activities (McRipley and Sbarra, 1967; Babior *et al.*, 1975; Klebanoff, 1980). Since chemiluminescence has been shown to be an indicator of oxidative metabolism triggered by the interaction of macrophages with particles such as bacteria (Allen and Loose, 1976; Nelson *et al.*, 1976), Blumenstock and Jann (1981) examined the chemiluminescence response of *Ity*^{r} and *Ity*^{s} peritoneal macrophage cultures as a measure of early extracellular bactericidal activities. However, these investigators found that there was no difference in the chemiluminescence response of *S. typhimurium*-infected macrophages of either genotype. Nevertheless, they were able to demonstrate that resistance of these mice to infection with *S. typhimurium* correlated with the intracellular bactericidal activity of their macrophages. Consistent with these findings were the recent results of Lissner *et al.* (1983). These investigators infected *Ity*^{s} and *Ity*^{r} macrophages with an avirulent, temperature-sensitive mutant of *S. typhimurium* which does not replicate at 37°C and demonstrated that the *Ity*^{r} macrophages killed

intracellular salmonellae more efficiently than Ity^s macrophages. Taken together, these data suggest that the Ity gene product in some way influences the degree of microbicidal activity within the macrophage.

Although such detailed bacterial growth kinetic experiments and in vitro macrophage cultures have not as yet been undertaken with mice which express Lps^n and Lps^d, some parallel observations have been made. O'Brien, Metcalf, and Rosenstreich (1982a) demonstrated that S. typhimurium is cleared from the bloodstream and taken up by RES tissues with equal efficiency in C3H/HeN (Lps^n) and C3H/HeJ (Lps^d) mice. Moreover, pre-treatment of C3H/HeJ mice with the gram-positive macrophage activator, BCG, markedly reduced the net splenic growth of S. typhimurium to the control levels observed in untreated C3H/HeN mice. Thus, it appears that the inability of C3H/HeJ mice to control early intracellular multiplication of S. typhimurium is not a result of impaired phagocytosis. These results suggest instead, that their macrophages can not be adequately activated. Nevertheless, even when the macrophages were activated with BCG, the mean time to death was only extended to 18 days.

Though the mechanism(s) by which Ity and Lps genes exert their control is not known, the preceding observations suggest that after uptake of salmonellae by macrophages, Ity and Lps gene products influence the early

rate of net bacterial multiplication. In normal *Ity*^{r} and *Lps*^{n} mice, the multiplication of *S. typhimurium* is slower than that in salmonella-susceptible *Ity*^{s} and *LPS*^{d} mice and plateaus about 5-7 days post-infection (Gröschel *et al.*, 1970; Hormaeche, 1979c), presumably because salmonella-specific acquired immunity is allowed to develop (Collins and Mackaness, 1968). In contrast, the macrophages from susceptible mice are apparently unable to curb bacterial replication and the mouse dies before specific immune mechanisms can develop. The observation that BCG-induced augmentation of C3H/HeJ macrophage effector function only extends the mean time to death, and does not cure the animal, suggests that other cell types may be operative later in the infectious process. Thus, the capacity to control the initial net growth rate does not ensure survival of the host later in the course of the infection but, if either the *Lps*^{d} or *Ity*^{s} allele is expressed, the mouse will die early in the infection. Whatever the mechanism(s) by which *Ity* and *Lps* genes control the early response to *S. typhimurium*, the products of these two genes are distinct since gene complementation occurs among the F_1 progeny derived from crosses between mice of these two genotypes (O'Brien *et al.*, 1980b).

Xid is an X-linked recessive allele carried by CBA/N mice and their hemizygous F_1 male progeny (F_1 males). This gene confers susceptibility to *S. typhimurium* on these mice (O'Brien *et al.*, 1979b) and a B-cell defect which

results in poor or absent antibody responses to a variety of both thymic-independent (TI; Scher et al., 1973; Mosier et al., 1977) and thymic-dependent (TD; Janeway et al., 1975; Scher et al., 1979) antigens; low pre-immune serum IgM and IgG_3 levels (Amsbaugh et al., 1972; Perlmutter et al., 1979); failure to form B lymphocyte colonies in soft agar (Kincade, 1977); and failure to be stimulated by anti-immunoglobulin (Sieckmann et al., 1978). Other investigators have demonstrated that these abnormalities in immune function are due to the absence of a late developing subpopulation of B cells which express the Lyb 3, 5, and 7 differentiation antigens (Huber et al., 1977; Ahmed et al., 1977; Subbarao et al., 1979). However, T-cell function is relatively normal as indicated by mitogen responses to Concanavalin-A, specific in vitro T-lymphocyte-mediated cytotoxicity, skin graft rejection (Scher et al., 1975), helper cell activity (Janeway and Barthold, 1975; Janeway et al., 1980) and salmonella-specific T cell-dependent delayed type hypersensitivity (O'Brien et al., 1981). In addition, these immune-defective mice appear to have relatively normal macrophages/accessory cells as judged by: 1) the capacity of these cells to act as antigen-presenting cells for trinitrophenyl (TNP)-KLH or TNP-Ficoll responses (Boswell et al., 1980); 2) the ability to release prostaglandins and lymphocyte-activating factor (LAF) in response to LPS (Rosenstreich et al., 1978); and 3) the

capacity to contain early net growth of S. typhimurium (O'Brien et al., 1979). Furthermore, many studies have investigated whether the xid gene influences the development of B cells directly or indirectly by providing the improper developmental milieu through its effects on other cells. Hence, Nahm et al. (1983) examined X chromosome expression in xid heterozygous female mice using alloenzymes of phosphoglycerate kinase-1 (PGK-1) as X chromosome markers. The X chromosome from the immune-defective parental mouse strain contained the xid gene and the $Pgk\text{-}1^b$ allele. In this manner, they determined which X chromosome was expressed in different cell populations since females were mosaic because of X chromosome inactivation. Therefore, cell populations directly affected by the xid mutation would not express the xid-bearing X chromosome, whereas those indirectly affected or unaffected would express this chromosome randomly. These investigators demonstrated that splenic B lymphocytes were nearly devoid of the alloenzyme encoded by the X chromosome bearing the xid gene while non-B lymphocytes from the spleen and from other tissues had a balanced representation of both chromosomes. These results suggested that xid affects B cells directly and not through their developmental milieu. In contrast, Teale (1983) found that the defect in F_1 male mice correlated with the stimulatory environment or, perhaps, T cells from immune-defective mice rather than the B cells. Scher et al. (1979) also studied

the relationship between the stimulatory environment and the X-linked immune defect. These investigators showed that the immune defect influenced the stimulatory environment since the environment of lethally irradiated xid mice was less effective in supporting an IgG antibody response to the TD antigen, sheep red blood cells (SRBC), than that of normal mice. However, when the responses of B cells from xid and normal mice were compared in the same environment, whether it was the defective or non-defective environment, the response of xid B cells was always significantly less than those of the immunologically normal B cells. These studies suggested that xid B cells have an intrinsic functional defect which is independent of the environmental defect. The factor(s) which influence this environmental defect are presently unknown. Nevertheless, it is possible to study the effect of the xid control of B lymphocytes since the environmental influence is distinct and probably secondary to the B-cell defect.

O'Brien et al. (1979) investigated the effect of the xid conferred B-cell defect on the susceptibility of mice to S. typhimurium. They found that, in contrast to Ity^s and Lps^d, the 10-day $L.D._{50}$ of S. typhimurium for immune-defective F_1 male mice was only 10-fold lower than for normal F_1 female mice. However, at 28 days post-infection, the $L.D._{50}$ of S. typhimurium for F_1 males was 1,000-fold less than for F_1 female mice. These studies

also demonstrated that the susceptibility of CBA/N mice to S. typhimurium, like their immune defect, was X-linked. In addition, linkage analysis revealed a close correlation between the presence of low serum IgM (as a marker for xid expression) and the susceptibility to S. typhimurium in F_2 and backcross mice derived from CBA/N parents. These studies suggest that the xid gene confers susceptibility to S. typhimurium late in infection. Moreover, immune-defective mice appear to carry the Ity^r and LPS^n resistance alleles (O'Brien et al., 1979; O'Brien et al., 1980) but, as noted above, the capacity to control the initial net growth rate does not ensure survival of the host late in the course of the infection. Therefore, mice which express the xid gene will eventually succumb to the infection even if the Ity^r and LPS^n alleles are both expressed. Additional studies by O'Brien et al. (1981) also support the hypothesis that the expression of the xid gene B-cell defect renders mice salmonella-susceptible. When compared to normal mice, F_1 male immune-defective mice were found to generate relatively low levels of serum anti-S. typhimurium antibodies after immunization with an acetone-killed and dried vaccine. Furthermore, passive transfer of immune serum from normal mice or reconstitution with bone marrow from normal mice markedly increased the resistance of immune-defective mice to infection for a 28 day period. Taken together, these data support the hypothesis that the susceptibility to salmonella in mice which express the xid

gene is a consequence of a defective anti-S. typhimurium antibody response. Moreover, these data suggest that B cells and their antibody products are important in the development of resistance to S. typhimurium late in the course of infection.

Collectively, the observations outlined in the previous paragraphs permit the development of a model (Figure 1; O'Brien, et al., 1982b) for the genetic control of murine resistance to S. typhimurium. The murine immune response to this bacterium can be divided into an early phase (<10 days after infection) and a late phase (>10 days after infection). The tenets of this model can be described as follows: after infection, the organisms are engulfed by splenic and hepatic macrophages. Early antimicrobial phagocytic processes such as oxygen-dependent events probably keep bacterial multiplication initially in check. However, the capacity of RES tissues to continue to restrict bacterial replication reflects the expression of salmonella response genes by the host. Thus, following phagocytosis, neither Ity^s nor Lps^d mice can control bacterial multiplication, and they die at an early stage. The ability to survive this apparently immunologically nonspecific phase of the infectious process not only reflects the animal's resistance to salmonella infections but also to Mycobacterium bovis strain BCG, Leishmania donovani, and Mycobacterium lepraemurium, which are presumably regulated

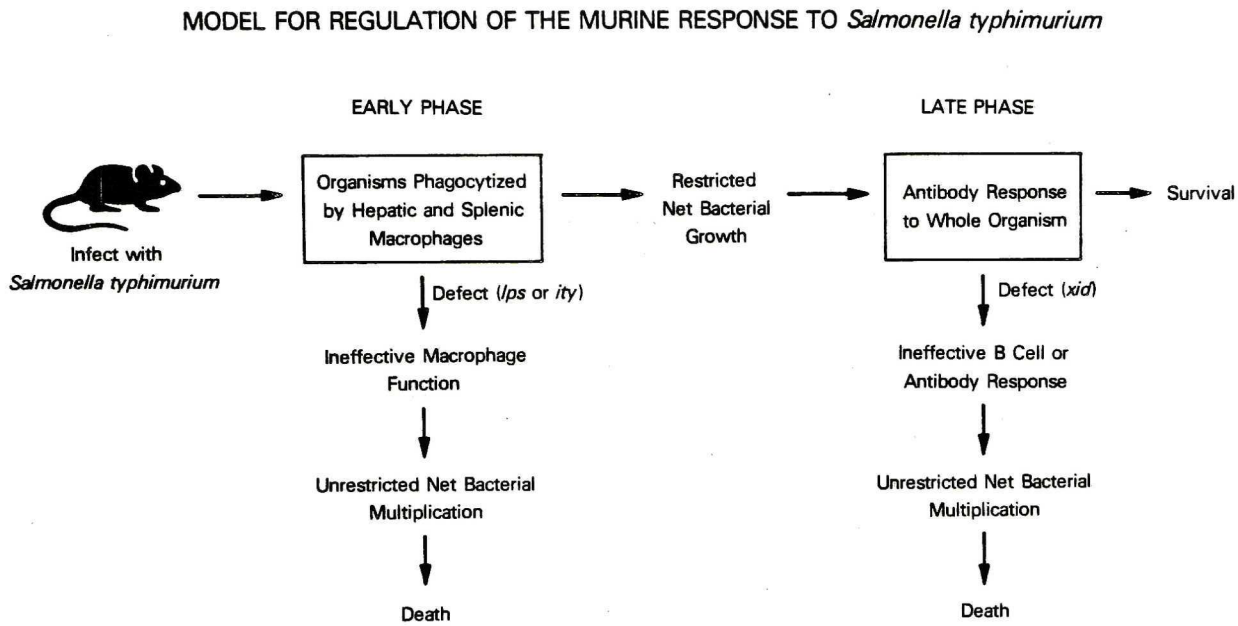
MODEL FOR REGULATION OF THE MURINE RESPONSE TO *Salmonella typhimurium*
EARLY PHASE
LATE PHASE
Infect with *Salmonella typhimurium*
Organisms Phagocytized by Hepatic and Splenic Macrophages
Restricted Net Bacterial Growth
Antibody Response to Whole Organism
Survival
Defect (*lps* or *ity*)
Ineffective Macrophage Function
Unrestricted Net Bacterial Multiplication
Death
Defect (*xid*)
Ineffective B Cell or Antibody Response
Unrestricted Net Bacterial Multiplication
Death

Figure 1.

by the same gene or a tightly linked complex (Skamene et al., 1982; Plant et al., 1982; Brown et al., 1982; Skamene et al., 1983). Interestingly, recent independent studies by Stach et al. (1984) and Blackwell et al. (see Stach et al., 1984) have directly demonstrated that both the Bcg (for BCG) and the Lsh (for Leishmania) genes, respectively, regulate the anti-mycobacterial and anti-leishmania activity of macrophages as has already been shown for Ity by Lissner et al. (1983).

Mice which survive the first phase of the infection, that is, mice which express the Ity^r/Lps^n phenotype, must develop immunologically specific mechanisms in order to survive the infection. Although, cellular mechanisms are probably important for survival at this late phase, production of salmonella-specific antibodies are also necessary. This is supported by the late deaths of the xid-conferred salmonella antibody-defective mice which can be prevented by the passive transfer of immune serum (as described above).

Recently, two additional S. typhimurium susceptibility genes were identified (O'Brien and Rosenstreich, 1983; A.D. O'Brien, B.A. Taylor, and D.L. Rosenstreich, manuscript in preparation). However, the basis for the salmonella-susceptibility of mice which express these genes has yet to be characterized. Therefore, the model outlined in Figure 1 may need revision as these genes and, perhaps, additional, as yet

unidentified genes, are more completely described.

This model suggests that, in addition to macrophages, humoral immunity plays an important role in resistance to S. typhimurium. This has been a point of contention in the past since many investigators believed that the specific protective immune response was essentially cell mediated and did not depend upon the presence of specific antibody. This view was supported by the studies of Mackaness, Blanden, and Collins (Mackaness et al., 1966; Blanden et al., 1966; Collins et al., 1966; Collins and Mackaness, 1968; Collins, 1969). These investigators reported that macrophages from S. typhimurium-infected mice had distinctly enhanced microbicidal properties when compared to normal mouse peritoneal macrophages. In addition, they showed that mice vaccinated with heat-killed organisms developed higher levels of humoral immunity than cellular immunity (Blanden et al., 1966; Collins and Mackaness, 1968; Collins, 1974). Furthermore, these vaccinated mice were not protected from intravenous challenge with salmonella. On the other hand, mice vaccinated with live vaccines, which presumably generate higher levels of cellular immunity (Blanden et al., 1966; Collins and Mackaness, 1968; Collins, 1974), were protected. Moreover, these studies by Mackaness and his colleagues showed that passive transfer of serum from actively infected or vaccinated animals increased the

capacity of the host to clear organisms from the blood but did not interfere with their subsequent multiplication in the tissues to any significant extent. This inability to passively transfer protection with immune serum was verified by other groups (Hobson, 1957; reviewed by Eisenstein and Sultzer, 1983). Finally, Collins and Mackaness demonstrated that protection with living vaccines involved concomitant development of antigen-specific delayed hypersensitivity (1968) and that T-cell depleted mice were incapable of developing anti-salmonella immunity or delayed hypersensitivity (Collins, 1974). Thus, these investigators concluded that the primary mechanism in the development of resistance to salmonella infections is cellular rather than humoral, and they perceived that the development of immunity to S. typhimurium proceeded as follows: salmonellae activate T cells and these stimulated T cells release macrophage-activating lymphokines. The macrophages, thus activated, are capable of enhanced bactericidal activities which result in the ultimate destruction of the infecting organisms. The disregard of these investigators for a role for humoral immunity in the development of resistance to salmonella infection was based primarily on their studies which showed the inability to protect against lethal infection by passive transfer of immune serum or by vaccination with killed vaccines. However, the validity of these findings remains controversial since innately resistant, outbred mice were

used in those studies where immune serum failed to provide protection. Hence, a sizable infecting dose was necessary to cause lethal infections in the resistant host. This antigen load may have been too overwhelming for host defenses, even in the presence of passively administered antibodies. Therefore, these studies may not fairly evaluate the role of antibodies in the development of protective immunity.

Although the studies of Mackaness, Collins, and Blanden support the theory that cell-mediated immunity is essential in the control of salmonella infections, which is still consistent with the proposed model for resistance (Figure 1), several reports support the importance of humoral immune mechanisms as well. Indeed, Jenkin, Rowley, and Auzins (1964) demonstrated that multiple injections of serum from mice immunized with an avirulent strain of S. typhimurium could protect mice for a period of 28 days against challenge with virulent S. typhimurium. In addition, many laboratories have found that active immunization with killed vaccines such as acetone or alcohol killed bacteria (Jenkin and Rowley, 1965; Angerman and Eisenstein 1978, 1980) and deoxycholate extracted or heat killed bacteria (Herzberg et al., 1972; Ornellas et al., 1970), as well as active immunization with bacterial components such as ribosome-rich extract, LPS (Angerman and Eisenstein, 1978, 1980), O-antigen (Svenson et al., 1979)

and porin protein (Kuusi et al., 1979) can provide protection. Some of these bacterial components were also shown to be protective in passive immunization studies. Svenson et al. (1981) purified several saccharides representative of the O-antigenic polysaccharide chain of S. typhimurium (O:4,12). Tetra-, octa- and dodecasaccharides were used as haptenic groups covalently linked to bovine serum albumin. They demonstrated that rabbit serum antibodies elicited by the saccharide-protein conjugates could passively protect mice for a 30 day period against lethal challenge with S. typhimurium (O:4,12) but not against challenge with S. enteritidis (O:9,12). Another outer membrane component, porin protein, extracted from a rough mutant of S. typhimurium has similarly been shown to confer passive immunity (Kuusi et al., 1979), although a later report (Kuusi et al., 1981) suggested that this protection was elicited by antibodies which recognized a conformational determinant between the native porin trimer and LPS. Furthermore, Svenson and Lindberg (1978, 1979) demonstrated that their salmonella O-antigen-protein conjugates elicited high titers of specific anti-carrier protein antibodies as well as anti-O antibodies in rabbits. Ostensibly, O-antigenic determinants coupled to another outer membrane component could provide a more potent immunogen. Svenson et al. (1979) indeed found that immunity directed against both the O-antigen and the porin conferred better protection than immunity against either

antigen alone. The O-antigen-porin conjugate conferred protection both actively and passively. Recent studies by O'Brien, Scher, and Metcalf (1981) demonstrated that (CBA/N x DBA/2)F_1 male mice which express the xid immune defect could be protected from lethal infection by immune sera from immunologically normal F_1 female mice. Moreover, Morris et al. (1976) used immunosuppressive agents to study the relative importance of T and B lymphocytes in the protection of mice vaccinated with an avirulent mutant of S. typhimurium (strain G30D). They found that mice treated with the B-cell suppressive agent, cyclophosphamide, could not even control multiplication of G30D and were eventually killed by this vaccine strain. By contrast, another group of mice were treated with the T- and B-cell suppressive agent, anti-lymphocyte serum (ALS). This group of mice then received immune serum from G30D vaccinated mice to supplement the ALS-induced suppression of their humoral immune response. Consequently, these mice were considered to be suppressed in T-cell functions only. Vaccination of the T-cell suppressed group of mice with G30D was able to provide protection after challenge with a lethal strain of S. typhimurium. Finally, Hochadel and Keller (1977) obtained partially purified lymph node B cells (which probably contained macrophages) and thymic T cells from mice shown to be immune to S. typhimurium. They demonstrated that normal mice injected with the immune B

cells showed a significant reduction in the number of challenge organisms recovered from their blood, spleen, and liver along with a better rate of survival from the challenge dose of S. typhimurium than did mice injected with the immune T cells.

Taken together, these data suggest that acquired antibody or functional B lymphocytes are essential in resistance against a virulent challenge infection with S. typhimurium. Although antibody appears to be important for survival against murine typhoid, many questions remain: 1) what is the role of antibody in protection? 2) what is the heavy chain class specificity of the protective anti-salmonella response? 3) which antigenic determinants are important in the development of protective immunity?

The adult mouse contains a complex, heterogeneous, B-cell population which is responsive to a wide array of antigenic determinants. To dissect the antibody response to S. typhimurium and understand the role of specific B cells and their antibody products in the immune response, a study of the antibody responses of individual salmonella-specific B cells is required. To date, there have not been any detailed analyses of B cells and their antibody products which are specific for a bacterial organism. Such studies would provide insights into those subsets of B cells responsive to natural antigens, and, in addition, provide an experimental approach for the analysis of the humoral immune response against other infectious agents.

Thus, the purpose of this thesis is to: 1) design a system which will permit an assessment of the frequency of B cells that participate in the primary response to S. typhimurium; 2) compare this frequency of B cells which is responsive to a natural antigen with those frequencies obtained with small, synthetic haptenic determinents in previous studies; and 3) to characterize the antibody products of individual salmonella-specific B cells by heavy chain class analysis and fine specificity analysis. These studies should provide insights into the immune mechanisms involved in the response to S. typhimurium and other infectious disease organisms. The validity of this kind of study which examines the repertoire of B cells responsive to a particular antigen rests on an understanding of the fundamental principles of specific antibody formation.

The first detailed theory for the production of specific antibodies was proposed by Ehrlich in 1900 (Ehrlich, 1900). He postulated that the surface of immunocompetent cells was covered with pre-formed antibody-like receptors for antigen ("side chains") which were identical to the antibody product found in serum. He envisaged that antigen, specifically bound to a complementary "side chain," in someway stimulated the cell to synthesize and secrete new "side chains." However, at that point in time, it was difficult to accept that antibodies elicited to an extremely wide variety of

antigens could all pre-exist. Thus, Ehrlich's Side Chain Theory was abandoned. Instead, the Instructive Theory of antibody formation, suggested by Mudd (1932), Pauling (1940), and Karush (1962), gained popularity. This theory predicted that antigen acted as a template over which antibody molecules were molded to form a complementary configuration. However, when it became evident that the specificity of the antibody combining site is inherent in the amino acid sequence of the antibody which is encoded by nucleotide sequences in the DNA (Haber, 1964; Whitney and Tarfold, 1965; Jaton et al., 1968), the Instructive Theory was disregarded.

A. The Clonal Selection Theory

The advancement of the Clonal Selection Theory (Jerne, 1955; Burnet, 1959) has since provided the theoretical framework for understanding antibody specificity. The essence of this theory, as stated by Burnet (1959), is that: "No combining site is in any evolutionary sense adapted to a particular antigenic determinant. The pattern of the combining site is there and if it happens to fit, in the sense that the affinity of absorption to a given antigenic determinant is above a certain value, an immunologically significant reaction will be initiated." Briefly, Burnet's postulates can be stated as follows: 1) the receptor of an antibody-forming cell precursor is identical in specificity to the eventual

antibody product of that cell; 2) each precursor cell is committed to the synthesis of one and only one antibody specificity prior to interaction with antigen; and 3) the progeny of that precursor remains committed to that specificity. The major tenets of this theory have been extensively confirmed experimentally (reviewed in Sigal and Klinman, 1978). With regard to this thesis, if these tenets had not been verified, then the assessment of the antigen-specific repertoire made herein would be meaningless.

B. Assessment of the B-Cell Repertoire

Evidence for the Clonal Selection Theory has established the existence of a diverse repertoire of B-cell precursors each of which is committed to the synthesis of antibodies of a unique specificity, and each of which expresses antigen-specific receptors which are immunoglobulin in nature. Thus, an assessment of the B-cell repertoire which is specific for a particular antigen can be legitimately fulfilled.

One way in which to estimate the fraction of the total repertoire which is responsive for a given antigen is by the enumeration of B cells with surface receptors which can bind that antigen. Antigen binding cells have been detected by autoradiographic techniques, rosette formation, enzymatic reactions and fluorescent antibody techniques. In this manner, Naor and Sulitzeanu (1967)

determined that 0.06% of normal mouse spleen cells were capable of binding ^{125}I-bovine serum albumin (BSA). Several laboratories, using radiolabeled antigens, obtained similar frequencies (Ada and Byrt, 1969; Mandel et al., 1970; Dwyer and Mackay, 1970; Humphrey and Keller, 1970). However, the actual number of antigen binding cells counted depends on the concentration of labeled antigen in the reaction mixture (Byrt and Ada, 1969; Ada, 1970; Dwyer and Mackay, 1972), the specific activity of the label (see Roelants, 1972), and the exposure time for autoradiography (Ada, 1970; Dwyer and Mackay, 1972). Frequencies that have been obtained by rosetting techniques, whereby antigen-coated erythrocytes form clusters around lymphoid cells, apparently had not distinguished rosette-forming B cells from rosette-forming T cells, (see Roelants, 1972; Lefkovits, 1974). Although the above radiolabeled antigen-binding experiments did not indicate whether the reactive cells were T or B cells, Dwyer et al. (1971) observed frequencies of ^{125}I-flagellin-binding cells in athymic nude mice similar to those in normal mice.

Another approach used to enumerate antigen binding cells involved substrate cleavage by B-galactosidase (GZ) specifically bound to cells of various murine lymphoid organs (Sercarz et al. 1971; Deluca et al., 1974). The GZ-binding cells were detected by a color change reaction observed under light microscopy. Whereas the frequency of antigen binding cells, as measured by radiolabeled antigen

or rosetting techniques, ranged between 0.001% - 0.1% of the normal mouse spleen cells, the estimate for GZ-binding cells was much greater, 2% for spleen cells. It is not quite clear what the reasons are for the discrepant results, although, these latter experiments were done under antigen saturating conditions while the former experiments were not.

It is clear, however from these different studies, that, depending on the antigen and the method used, repertoire estimates differed significantly. Subsequent studies showed that antigen binding cells did contain the precursors for antibody-forming cells. Ada and Byrt (1969) showed that the pre-treatment of normal spleen cells with ^{125}I-flagellin of very high specific activity abolished a subsequent antibody response to a homologous flagellin but not to a flagellin of a heterologous strain. Moreover, Wigzell and Mäkelä (1970) passed a mixture of lymph node, spleen and bone marrow cells from non-immune mice through an ovalbumin (OVA)-coated column. The column passed cells were transferred into lethally irradiated mice which were subsequently challenged with OVA. These recipient mice failed to produce serum anti-OVA responses. Thus, the specific depletion of a population of antigen binding cells with the concomitant elimination of antibody responsiveness to that antigen suggested that antigen binding cells did contain the capacity to elicit a primary antibody response.

Studies by Haas (1975) confirmed and extended these findings. Normal mouse spleen cells were fractionated on plates coated with dinitrophenyl (DNP)-gelatin. DNP-specific cells were isolated by first melting, then digesting, the gelatin with collagenase. Cells which did not bind the haptenated gelatin did not give rise to DNP-specific plaque-forming cells following an *in vitro* stimulation with DNP-polymerized flagellin (DNP-POL), whereas cells which had bound to the gelatin did generate an antibody response. On the other hand, antigen binding is not always equivalent to antibody responsiveness, albeit, antigen binding is necessary for stimulation. Klinman *et al*. (1976) showed that only 8% of the DNP-binding cells were actually stimulated to produce anti-DNP antibody. In addition, the vast majority of cells which specifically bound to DNP-gelatin at high antigen concentrations were not stimulated by that antigen since increasing the stimulating antigen concentration did not increase the number of responding antigen-specific, primary or secondary B cells. Moreover, increasing antigen concentrations did not permit the stimulation of B cells whose antibody product was of relatively low affinity (Klinman, 1972). Thus, it appears that, while low affinity B cells may bind antigen, only those B cells with receptors of high affinity for the bound antigen can be sufficiently stimulated to produce antibody. Conversely, binding alone is sufficient for B-cell triggering by some antigens such as large

polymeric antigens like trinitrophenylated Brucella abortus (TNP-BA) (Mond et al., 1980; Morrissey et al., 1981), TNP-lipopolysaccharide (TNP-LPS) (Jacobs and Morrison, 1975; Chused et al., 1976), or B-cell mitogens (Coutinho et al., 1974; Möller, 1975). Further, for other antigens, such as phosphorylcholine (PC), the frequency of antigen binding cells is equivalent to the precursor cell frequency (Klinman et al., 1976). Therefore, the frequency of antigen binding cells can only give a relative estimate of the number of physiologically relevant B cells involved in the specific antibody response to an antigen.

Other methods for estimating the fraction of cells specific for a given antigen test the capacity of precursor cells to produce antibody. The in vivo splenic focus technique (Kennedy, et al., 1966; Playfair et al., 1965) was used in early attempts to obtain an assay for the precursors of antibody-forming cells. In this system, small numbers of donor spleen cells and antigen were injected into lethally irradiated syngeneic recipients whose spleens were removed and cut into small fragments several days later. Each fragment was presumed to contain a single SRBC-specific precursor cell which, in the presence of antigen (SRBC), would proliferate and differentiate to give rise to a clone of antibody-forming cells. Antibody would then diffuse out from the fragment into a layer of agar containing sheep erythrocytes and

complement-dependent lysis would be observed around those fragments which contained antibody-forming cells (a hemolytic focus). Indeed, these studies showed that the number of hemolytic foci in a recipient spleen was directly proportional to the number of cells injected. However, studies from other laboratories indicated that this was not always the case. Many foci were found to contain more than one B cell (Vann and Campbell, 1970; Luzzati et al., 1970) and, in addition, the identity of the limiting cell type was unclear.

Cudkowicz and co-workers (1970) approached this problem using a cell transfer, limiting dilution system in which graded numbers of cells were injected into syngeneic, irradiated recipients followed by immunization and assay of an all-or-none antibody response. Henceforth, they transferred limiting doses of bone marrow cells, as a source of B cells, along with a large constant number of thymus cells. The plot of the number of splenic plaque forming cells (PFC) specific for SRBC versus the number of injected bone marrow cells followed a Poisson distribution. Thus, since B cells were found to be the limiting cell type, this study allowed an estimate of the frequency of SRBC-specific precursor cells, which was determined to be 3.3×10^{-6} bone marrow cells.

Using a different approach, Möller and Michael (1971) transferred graded numbers of spleen cells along with a thymus independent antigen, LPS, to ensure that only

the B cells were limiting. The anti-LPS PFC response also followed a Poisson distribution; however, their estimated precursor frequency of 1 x 10^{-5} B cells specific for LPS may have been too high due to non-specific triggering of B cells by LPS which also acts as a B-cell mitogen.

Using a microculture method, Quintans and Lefkovits (1973) titrated a range of spleen cells per microculture from nude mice, as their source of B cells, in the presence of a constant number of allogeneic spleen cells which provided a functional excess of T cells. The frequency of precursor B cells specific for SRBC, calculated by means of Poisson statistics, ranged between 0.9 - 2.0 x 10^{-5}. The linear dose-response kinetics demonstrated that the B cell was limiting in these cultures. In a similar system, Cosenza (1975) and coworkers determined that the frequency of PC-specific B cells responsive to the thymus-independent antigen Streptococcus pneumoniae strain R36A was 2 x 10^{-5}.

The in vitro splenic focus system, which is a modification of the in vivo technique developed by Kennedy et al. (1965) and Playfair et al. (1965), has also been used to isolate individual antigen-specific B cells and to analyze the antibody product derived from each clone. Since a modification of this technique which was originally described by Klinman (Klinman and Aschinazi, 1971; Klinman, 1972) has been employed extensively in the studies

described in this thesis, it will be described in some detail. Limiting numbers of donor spleen cells, as a source of B cells, are transferred into lethally irradiated syngeneic recipients which have been primed eight weeks previously with a protein carrier, emulsified in complete Freund's adjuvant. Recipient spleens are removed 16-18 hours after donor cell transfer, sliced into 1mm cube fragments and each fragment placed individually into wells of a microtiter plate. Fragments are incubated for 3 days in medium which contains a stimulating dose of a hapten conjugated to the appropriate carrier. Anti-hapten antibody is assayed 9-14 days after stimulation by a solid-phase radioimmunoassay which has been shown to be capable of detecting as little as 0.5 ng of antibody (Klinman and Taylor, 1969). This system is useful not only because the frequency of cells responsive to an antigen can be determined, but also because sufficient quantities of homogeneous antibody can be obtained to do subsequent assays for isotype, idiotype, isoelectric point or fine-specificity analysis. Thus, unique antibody molecules, (clonotypes), each elicited by the same antigen, can be identified and their precursor frequencies determined as well.

Monoclonality of the elicited antibody has been demonstrated in this system by restricted isoelectric focusing spectra (Klinman, 1972; Press and Klinman, 1973a; Klinman and Press, 1975b), idiotype analysis (Gearhart et

al., 1975a,b), equilibrium dialysis (Klinman, 1969, 1971a, 1972), regaining of antibody activity following homogeneous, but not heterogeneous, heavy-light chain recombination (Klinman, 1971c), and the all-or-none binding characteristics of anti-influenza monofocal antibodies to cross-reacting strains of influenza virus (Gerhard et al., 1975). Several studies have shown that the number of clones detected is linearly related to the number of donor cells transferred, even when donor cells are depleted of T cells (Klinman and Aschinazi, 1971; Klinman, 1972; Klinman and Doughty, 1973; Doughty and Klinman, 1973; Press and Klinman, 1973b). This suggests that only the B cell is limiting in this system. Further, primed recipient fragment cultures provide excess T cell and macrophage accessory cell help, thereby ensuring that B-cell responses are maximized. Moreover, B-cell stimulation in fragment culture closely simulates in vivo conditions, thus, the affinity, isotype, and clonotype of monoclonal antibodies detected in the splenic focus system closely reflects the in vivo serum response (Klinman, 1972; Gearhart, 1977).

Pierce and Klinman (1977) have demonstrated that carrier-specific enhancement can be maximized for B cells from non-immune donors when they are transferred to immunized recipients which are syngeneic at the H-2 locus but allogeneic at the immunoglobulin heavy chain locus. Such a donor-recipient combination apparently bypasses an

antibody-specific regulatory mechanism present in immunized recipients which specifically limits the stimulation of syngeneic, hapten-specific, primary B cells. The suppression of primary B cells is not due to the absence of appropriate carrier-specific helper T cells. H-2 identical and allotype distinct strain combinations of mice are particularly important in allowing primary B-cell responses to antigens for which carrier molecules are not readily available, such as responses to viruses and to bacteria.

In order to establish an absolute measurement of the B-cell repertoire to a given antigen using the splenic focus system, it is imperative to correct the frequency estimates for cloning efficiency. The cloning efficiency has been determined using two different experimental approaches (Klinman et al., 1976). The first method measured the increment in antigen binding cells in immunized and normal donor spleen cells, and compared this increment to that obtained from measurements of clonal precursors by in vitro splenic focus analysis. The efficiency of the system was found to be 3.9%. The second approach compared the number of PC-specific precursors in the splenic focus system to the frequency of PC-binding cells. These studies confirmed the efficiency to be 3.9%. Homing efficiency for both adult and neonatal, chromium-labeled, donor lymphocytes was determined to be 5%. Therefore, the cloning efficiency of 3.9% indicates that approximately 80% of the antigen-specific precursor B cells

which lodge in the recipient spleen are stimulated to detectable antibody-forming cell clones.

The splenic focus system has been employed to estimate the frequency of precursor cells specific for a variety of antigens (Klinman, 1972; Press and Klinman, 1974; Sigal et al., 1975; Klinman et al., 1976; Sigal, 1977; Cancro et al., 1978; Stashenko and Klinman, 1980). Moreover, B cells from a wide variety of sources can be stimulated to antibody production in this system such as fetal and neonatal B cells (Press and Klinman, 1973b); immune and non-immune B cells (Klinman, 1972); B cells from bone marrow, spleen, lymph nodes, blood, Peyer's patches, and pulmonary lymphoid tissue (Klinman, 1972; Metcalf and Klinman, 1977; Pierce et al., 1978; Gearhart and Cebra, 1979; Fuhrman and Cebra, 1981). This system also permits the identification and characterization of B-cell subpopulations (Metcalf and Klinman, 1977; Metcalf et al., 1980; Kenny et al., 1983).

C. Identification of Unique Antibody Specificities

In accordance with the tenets of the Clonal Selection Theory, it has been demonstrated that the specificity of the humoral immune response to any given antigenic determinant is derived from a heterogeneous population of B cells. Each B cell within the population produces a single antibody specificity (clonotype). Hence, in addition to determining precursor B-cell frequencies to

a particular antigen, the response to that antigen can be further characterized by identification of the unique antibody specificities which compose the heterogeneous antibody population produced in the response. A number of methods have been used to identify a clonotype or group of clonotypes and will be briefly reviewed herein. A more comprehensive review (Sigal and Klinman, 1978) deals with this subject in greater detail.

Gerhard (1976, 1977) analyzed monoclonal, secondary anti-influenza A, viral hemagglutinin (HA) antibodies derived in the splenic focus system on a panel of nine immunoadsorbents which included the homologous stimulating antigen, PR8, and eight other viruses of closely related HA type. These studies revealed 47 distinguishable cross reactivity patterns. Thus, this methodology was able to dissect the complex antigenic determinants on a single protein into its integral components, as well as, establish the wide array of clonotypes present in BALB/c mice capable of recognizing the PR8 HA.

This type of fine specificity analysis was extended to primary B-cell clones (Cancro et al., 1978) in which 36 distinguishable reactivity patterns were identified. It was observed that the reactivity patterns present in the primary B-cell repertoire was very similar, if not equivalent, to those present in the secondary repertoire. In addition, reactivity patterns of conventionally reared

mice were compared to those of germ free mice (Cancro et al, 1978) and athymic nude mice (Cancro and Klinman, 1980). All were found to be similar. Recently, comparisons between adult and neonatal repertoires and between repertoires of genetically distinct strains of mice have been examined by this analysis (Cancro et al., 1979; Cancro and Klinman, 1981; Cancro, 1982).

Fine specificity characterization of monoclonal or serum antibody has defined a number of clonotype subsets on the basis of the antibody's relative affinity for related haptens. When Imanishi and Mäkelä (1973) immunized C57BL mice with the hapten (4-hydroxy-3-nitrophenyl) acetyl (NP) on a protein carrier, the resulting anti-NP antibodies had a higher relative affinity for the structurally related haptens NIP, NNP, and NBrP than for the original immunogen, NP. These antisera, termed heteroclitic, were assessed by hapten inhibition of PFCs or inhibition of serum antibody binding to haptenated phage. Most mouse strains were found to produce non-heteroclitic antibody, that is, they had the highest affinity for the immunizing hapten. McMichael et al. (1975) demonstrated in experiments using congenic, recombinant inbred, and backcross mice that anti-NP heteroclitic antibodies have a strain-specific pattern in isoelectric focusing (IEF) and that these characteristics are all controlled by a Mendelian gene that is linked to the immunoglobulin heavy chain gene. The antibody defined by heteroclitic fine specificity analysis is a

heterogeneous population of molecules as demonstrated by IEF. Initial IEF studies had shown that one set of antibody bands (a spectrotype) was the predominant antibody in the C57BL anti-NP response (McMichael et al., 1975) but later studies have detected three to four additional spectrotypes (Mäkelä and Karjalainen, 1977). The validity of the fine specificity analysis as a tool to identify a group of clonotypes has been confirmed by the generation of an anti-idiotypic serum made against the purified C57BL anti-NP antibody. This anti-idiotypic serum reacts with all four spectrotypes.

DiPauli (1976) characterized the cross-reactivity patterns of murine IgM anti-Salmonella anatum LPS by their relative avidity toward heterologous LPS. Although, among different strains of mice, two phenotypes were identified by cross-reactivity patterns against LPS from S. cholera-suis, IgM antibodies from individual mice were shown to have a heterogeneous anti-LPS repertoire based on their reactivity toward the LPS from S. strasbourg and S. illinois.

As mentioned above, another method which has been used to identify unique antibody specificities is IEF which utilizes charge differences arising from disparities in the amino acid sequence of the variable (or constant) region. Kreth and Williamson (1973) used a spleen cell transfer system, together with the determination of the isoelectric

spectra of secondary IgG antibodies present in sera of recipient mice, to give an estimate of the number of different clones of antibody-forming cells recruited into a specific anti-4-hydroxy-5-iodo-3-phenacetyl (NIP) response. Their experiments were carried out in two stages. At different times after immunization with NIP-bovine IgG (BGG), limiting dilution experiments were performed to determine the smallest number of primed spleen cells that would give rise to only a single antibody-producing clone in a recipient mouse, as defined by IEF. After this donor cell inoculum was determined, all the cells from one donor spleen were distributed into a series of irradiated recipients at that dose of cells determined to give rise to a single monoclonal antibody. The isoelectric spectra of the sera of recipient mice were examined for the number of total and unique antibody molecules. They found that there was a preferential expansion of certain clonotypes early after priming, with the fewest repeats occurring around 70 days after priming. These data suggested that diversity is greatest at approximately ten weeks after immunization. A comparison between the sets of monoclonal antibody spectra derived from four different donor mice, expressed in the sera of irradiated recipients, showed a low, but significant, repeat frequency for individual spectra. Using this repeat frequency, these investigators were able to estimate the minimum number of different anti-NIP molecules potentially capable of being elicited by the

donor strain of mice. Although the relevance of Kreth and Williamson's estimation of the NIP-specific clonotype repertoire extends beyond the scope of this thesis, many aspects of their analyses merit brief discussion. First, the analysis assumed that 5×10^4 different clonotypes could be distinguished by their slab-gel IEF technique (Williamson et al., 1973). However, IEF studies of myeloma proteins which were known to have multiple amino acid differences revealed that IEF may not be able to discriminate as many clonotypes as Williamson et al. (1973) had originally assumed (Pink and Skvaril, 1975). Therefore, it would appear that only a minimum number of clonotypes were identified. It is equally possible they may have overestimated the number of antibody specificities. For example, the immunoglobulin produced by a single clone of cells is biosynthetically homogeneous, but post-synthetic changes generate microheterogeneity (Awdeh et al., 1970). Thus, variations in isoelectric spectra caused by post-synthetic modification may lead to recognition of false clonal identities. Moreover, differences in IgG subclasses may have also obscured identities.

Antibody from splenic fragment cultures have been analyzed by sucrose gradient IEF which allows high molecular weight immunoglobulins, such as IgM, to be focused (Press and Klinman, 1973) as well as IgG and IgA.

Accordingly, Klinman and Press (1975b) examined the clonotype repertoire of neonatal BALB/c mice for the haptens DNP and TNP. Further, Gearhart et al. (1975b) demonstrated that antibody from a single clone can express one idiotype in combination with a number of heavy chain classes. This methodology has overcome some of the problems encountered in Kreth and Williamson's (1973) studies since focused antibody clones can be simultaneously assayed for idiotype and heavy chain class to maximize clonal identification. Moreover, post-synthetic modification is minimized in vitro (Awdeh, 1970).

A very powerful tool for clonotype identification, idiotypy, employs antibodies specific for variable region determinants. Anti-idiotypic antibodies are useful both in searching for clonotypes that are shared among individuals of a strain and in exploring relationships among variable regions from different strains. For example, the immune reponse to PC in BALB/c mice had been reported to be markedly restricted, both in immunoglobulin class and variable region antigenicity (Sher and Cohn, 1972; Cosenza and Köhler, 1972a). In addition, twelve naturally occurring plasmacytoma proteins with binding specificity for PC have been found in BALB/c mice (Cohn et al., 1969; Potter and Lieberman, 1970). Antibody raised in A/He mice that is specific for the variable region of two of these myeloma proteins (TEPC 15 and HOPC 8) reacts with naturally occurring antibody produced in BALB/c mice in response to

immunization with R36A bacteria (Cosenza and Köhler, 1972a,1972b). Gearhart et al. (1975a) demonstrated that 75% of PC-specific B cells in splenic fragment cultures shared the TEPC 15 idiotype as indicated by the inhibition of binding of labeled TEPC 15 by the monoclonal antibody. Further, all the TEPC 15 monoclonal antibodies had identical IEF spectra, even in clones producing anti-PC antibody of more than one isotype (Gearhart et al., 1975b). Therefore, it was possible to determine the average repeat frequency for this single TEPC 15 clonotype in non-immune BALB/c mice which was found to be 1 per 60,000 B cells (Sigal et al., 1975).

While the majority of clones produced antibody of the TEPC 15 idiotype, approximately 25% produced anti-PC antibody of other idiotypes (Gearhart et al., 1975a; Sigal et al., 1975). Gearhart et al. (1977), in addition to the A/He anti-TEPC 15 sera (M anti-T15), prepared a rabbit anti-idiotypic antibody to TEPC 15 (R anti-T15). Monoclonal anti-PC antibodies from four different inbred mouse strains, generated in the in vitro splenic focus system, were quantitatively analyzed for the presence or absence of M anti-T15 and R anti-T15 reactivities using a radioimmunoassay inhibition technique. Four types of antibodies could be distinguished in this manner including the dominant TEPC 15 clonotype. Therefore, the use of two anti-idiotypic reagents indicated that non-TEPC 15 idiotype

monoclonal antibodies comprised several distinct clonotypes and that the TEPC 15 clonotype was present at much lower frequencies in AKR mice than in BALB/c mice (1/400,000 and 1/60,000,respectively) and is completely absent in C3H and A/J mice. Thus, the expression of the TEPC 15 clonotype at the precursor level is variable.

Finally, the most definitive way in which to identify unique antibody specificities is amino acid sequence analysis. However, very few methodologies provide sufficient quantities of antigen-specific antibody. The development of hybridoma techniques (Köhler and Milstein, 1975) which expand clones of antibody-producing cells and the advent of micro-sequencing systems will eventually allow such a detailed analysis. However, to date, sequencing is not routinely employed.

It should be clearly stated at the outset that there are certain limitations in any attempt to analyze the B-cell repertoire for unique antibody specificities. Firstly, in non-immune animals, identification of unique specificities may be extremely difficult since most are represented by a small proportion of the B-cell population. Secondly, dominant clonotypes may skew the true representation of specificities. For example, clonotypes represented by myeloma proteins with antibody specificity or clonotypes invariably expressed in large amounts in serum as the major antibody responding to a given antigen may not be truly representative of the primary B-cell

repertoire. Moreover, it is not known what proportion of the total potential of the repertoire is expressed by an individual at a given point in time. Hence, only part of this potential may be expressed at any point in time and may vary in expression from one point to the next within an individual's lifetime. Finally, it is not known to what extent environmental factors influence the potential extensiveness of the B-cell repertoire responsive to a particular antigen.

In summary, the purpose of this thesis is to estimate that fraction of the total CBA/Ca primary B-cell repertoire which is responsive to S. typhimurium and to critically analyze the antibody product of individual salmonella-specific B cells for isotype and fine specificity. In addition, it is also the aim of this thesis to compare the reactivity patterns and frequency of primary salmonella-specific B cells in these normal, innately resistant mice to those of the salmonella-susceptible, anti-S. typhimurium antibody-defective CBA/N mice.

In the present study, the splenic focus system has been modified in order to analyze the S. typhimurium strain TML (TML)-specific B-cell repertoire. The results of this study define conditions for obtaining monoclonal responses to acetone-killed and dried TML in these two strains of mice. The frequency of primary TML-specific splenic B

cells in CBA/Ca mice is extremely low when compared to the frequencies obtained with many chemically defined haptens and only a small proportion of these B cells are specific for the major Salmonella surface antigenic determinant, the LPS molecule. In contrast, the frequency of memory TML-specific cells is several orders of magnitude higher than the primary frequency and the majority of these clones are specific for LPS. Interestingly, CBA/N mice express no primary or secondary TML-specific precursors. However, after three immunizations, the CBA/N tertiary B-cell frequency is similar to that of primary CBA/Ca TML-specific B cells. In addition, the frequency of these B cells directed against the LPS molecule is intermediate between that of primary and memory CBA/Ca anti-LPS antibody producing clones. The isotype profile and fine specificity analysis of TML-specific clones not only reflect differences among all three B-cell subsets but indicate an abnormal anti-S. typhimurium antibody response in CBA/N mice.

II. Materials and Methods

A. Animals

Six to eight-week old CBA/CaJ, A/J, AKR/J, and B10.BR mice were obtained from Jackson Laboratories, Bar Harbor, ME. CBA/CaHN mice were obtained from the National Institutes of Health (NIH), Bethesda, MD. Eight to ten-week old CBA/N mice were obtained from Dominion Laboratories, Dublin, VA, or the NIH. The two sources of CBA/Ca mice were used interchangeably. C3H/HeN mice were obtained from Charles River Laboratories, Wilmington, MA.

B. Antigens

Salmonella typhimurium strain TML (TML) was originally isolated from a patient with salmonellosis (Gianella et al., 1971). Organisms were isolated on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI) plates grown at 37°C.

Acetone-killed and dried (AKD) bacteria of TML were prepared by the method of Landy (1953). Bacterial lawns of TML were grown on TSA plates, and the organisms were suspended in water (5 ml/plate). Cultures were harvested in a small volume of water. The organisms were precipitated after stirring with 2 volumes of acetone (HPLC Grade, Fisher Scientific Co., Fair Lawn, NJ) for 2-3 hours. The acetone was poured from the settled organisms and replaced with fresh acetone. The above acetone precipitation procedure was repeated 3 times. The final

bacterial precipitate was collected on a Buchner Funnel, thoroughly washed with acetone, and dried by air suction through the bacterial mass. Final traces of moisture were allowed to dry by spreading the powdered organisms on Whatman #2 paper overnight. The dried AKD-TML preparation was re-suspended in 0.15M phosphate buffer, pH 7.2 (PB). The absorbance was read at 600 nm (A_{600}) on a Gilford Spectrophometer 250 (Gilford, Gaithersburg, MD) and its concentration determined from a standard curve. The standard curve was constructed by relating the A_{600} absorbance with the concentration of varying dilutions of an AKD-TML preparation kindly supplied by Dr. Samuel Formal, Walter Reed Army Institute of Research, (WRAIR) Washington, D.C. The concentration of the WRAIR AKD preparation was previously established by empirical determination of its nitrogen content. The WRAIR AKD-TML vaccine, which was prepared by the same procedure as described above, was used in most studies. Therefore, the use of AKD-TML will refer to the WRAIR preparation unless otherwise noted.

Other inactivated TML antigens were prepared from bacteria grown overnight in Penassay broth (Difco) at 37°C with shaking. Overnight cultures were subcultured for two hours (early log phase) in Penassay broth. A cell count was determined by making serial 10-fold dilutions of a portion of the suspension and plating on TSA plates.

Colonies were counted one day later, and the total number of cells in the culture was related to the final suspended volume of killed bacteria. Immediately after a sample was taken for cell counts, 10 ml of the broth culture were centrifuged and washed twice in PB. The washed pellets were re-suspended in 10 ml of PB and treated in one of three ways: 1) heat-killed (Heat-TML) at 56°C in a water bath for 90 minutes with swirling at 30 minute intervals; 2) ultraviolet-irradiated (UV-TML) overnight at a distance of 16 cm from a 30 watt Germicidal UV lamp tube (Thomas Scientific, Philadelphia, PA). The 10 ml subculture was irradiated in a 60 mm plastic petri dish uncovered and re-suspended occasionally with a sterile pasteur pipet; or 3) glutaraldehyde-killed (Glut-TML) by the following method of Lowrie et al. (1979). The washed culture was re-suspended in 10 ml ice-cold sterile 2.5% (v/v) glutaraldehyde for 15 minutes at room temperature, washed twice in sterile 0.88M sucrose and then twice in sterile PB. All preparations were transferred to sterile graduated tubes after the inactivation procedure. Those preparations which evaporated during the inactivation process were brought back to the original volume (10 ml) with sterile PB. To ensure that each preparation was completely inactivated, 0.1 ml of each preparation was tested for growth on TSA plates.

Lipopolysaccharide (LPS) from TML was prepared by the procedure of Romeo et al. (1970). This phenol-water

extract of TML LPS is a standard laboratory reagent which has been shown to be free of lipid A-associated protein by a mitogenesis assay (Rosenstreich and Glode, 1975) performed with C3H/HeJ spleen cells. The B cells from these mice proliferate poorly in the presence of protein-free preparations of LPS (Sultzer and Nilsson, 1972), but blast formation is observed when C3H/HeJ spleen cells are incubated with LPS preparations which contain lipid A-associated protein (Sultzer and Goodman, 1976). The stimulation indices for duplicate cultures of C3H/HeJ spleen cells exposed to 0.1, 1 and 10 ng of TML LPS were 1.2, 1.5, and 2.0, respectively (Metcalf and O'Brien, 1981). In contrast, the stimulation index for C3H/HeJ spleen cells exposed to 10 ng of a butanol extract of E. coli strain 235 LPS, which contains lipid A-associated protein (Morrison and Lieve, 1975) was 10 (Metcalf and O'Brien, 1981).

Streptococcus pneumoniae type 3 was a gift of Dr. John Robbins, Bureau of Biologics, Bethesda, MD. S. pneumoniae was grown from a single isolated colony on blood agar plates (Difco) for 16 hours at 37°C with CO_2.

S. typhimurium LT-2 and the LPS-deficient mutants his-642 (Ra), HN202 (Rc), SL1004 (Rd1), SL1181 (Rd2), and TH2168 (Re) derived from it were the gift of Dr. J. K. Spitznagel, Emory University, Atlanta, GA. Organisms of each strain were isolated on TSA plates grown at 37°C.

Porin preparations were made from derivatives of S. typhimunium LT2. The mutant SH5551 and the mutant SH6017 which produce respectively, only the 36K and 34K protein (Ames et al. 1974) were kindly provided by Dr. Hiroshi Nikaido, University of California, Berkeley, CA. Porins were isolated and purified according to the method of Tokunaga et al. (1979a). Cells were grown in Penassay broth with the addition of NaCl to a final concentration of 1.5%. About 50 g of wet weight cells, harvested during late log/early stationary growth phase, were re-suspended in 5 volumes of 50mM Tris-HC1 buffer, pH 6.8 and sonicated twice on a Sonifer Cell disruptor, model W185 (Heat Systems-Ultrasonics, Inc., Plainview, NY) at 70 watts, passed 2 times through a French pressure cell (American Instrument Co., Silver Spring, MD) at 9,000 p.s.i. at 0°C, then sonicated once more. The extracts were centrifuged at 45,000 rpm (Sorvall OTB-50B Ultracentrifuge, TI50 rotor) for 80 minutes. The membrane fraction was re-suspended in 50mM Tris-HC1 buffer, pH 6.8 containing 1% sodium dodecyl sulfate (SDS) and incubated with gentle shaking at 37°C overnight. The peptidoglycan-associated SDS-insoluble material was collected by centrifugation at 45,000 rpm for 80 minutes and re-suspended in 50mM Tris-HC1 buffer, pH 6.8 containing 5mM EDTA and 1% SDS. After overnight incubation at 37°C, the insoluble material (crude porin extract) was collected by centrifugation. The crude porin extract was then washed twice by incubating in 50mM Tris-HC1 buffer, pH

6.8, containing 0.1% SDS for 2 hours at 37°C and then in 50mM Tris-HCl buffer, pH 6.8 containing 0.1% SDS and 3mM sodium azide for 1 hour. The crude porin extract was finally re-suspended in 50mM Tris-HCl buffer, pH 7.0, containing 1% SDS, 0.4M NaCl, 5mM EDTA, 0.05% 2-mercaptoethanol (2-ME) and 3mM sodium azide (SDS/salt mixture) and incubated at 37°C overnight. The soluble fraction was collected after centrifugation, concentrated by dialysis against dry Ficoll 400 (Sigma, St. Louis, MO), and applied onto a Sepharose 4B (Sigma) column (2.5 x 90 cm), which was equilibrated with the SDS/salt mixture. The protein peak was monitored by absorbance of the eluted material at 280 nm. Stained gels and densitometer tracings of stained gels after electrophoresis of the peak fractions showed that the majority of fractions contained a single protein band. Protein concentration at each purification step was determined by the method of Lowry et al. (1951).

Polymerized flagellin, prepared by the method of Ada et al. (1964) from S. typhimurium strain 7 was a gift of Cdr. Shannon Stewart, Naval Medical Research Institute (NMRI), Bethesda, MD. It was shown to contain 5.0% LPS by the KDO analysis of Karkhanis et al. (1978).

Purified lipid A from S. minnesota Re 595 was purchased from Calbiochem-Behring Corp., La Jolla, CA. This preparation contains 0.16% KDO.

C. Polyacrylamide Gel Electrophoresis

S. typhimurium outer membrane proteins were heated for 2 minutes at 100°C in a sample treatment mix that contained 0.0625M Tris-HC1 (pH 6.8), 2% SDS, 10% glycerol, 5% 2-ME and 0.001% bromophenol blue as the dye. The proteins were analyzed in the discontinuous buffer system described by Laemmli (1970). Separation, polyacrylamide slab gels, 0.75 mm thick, had an acrylamide: bis ratio of 30:0.8 and were polymerized chemically by the addition of 0.03% tetramethylethylene-diamine (TEMED) and 0.05% ammonium persulfate (0.05% and 0.1%, respectively, for stacking gels). The separation gels contained 0.375M Tris-HC1 (pH 8.8), 0.1% SDS, and 10% acrylamide. The stacking gels contained 0.125 M Tris-HC1 (pH 6.8), 0.1% SDS, and 5% acrylamide. Samples of 10-20 ul containing about 10 ng of protein were applied onto the top of the stacking gel. Electrophoresis was carried out with a current of 20 milliamperes per gel until the bromophenol blue marker reached the bottom of the gel, about 2-1/2 hours. Following electrophoresis, the gels were stained with Coomassie brilliant blue according to a modification of the method of Fairbanks et al. (1971). The gels were placed into a glass dish containing a solution of 25% isopropyl alcohol, 10% acetic acid, and 0.025% Coomassie blue for 1/2 to 1 hour with rocking. The gels were next incubated in a solution containing 10% isopropyl alcohol, 10% acetic acid and 0.025% Coomassie blue for 1/2 hour with rocking,

followed by incubation in a solution of 10% acetic acid and 0.025% Coomassie blue for a minimum of 1/2 hour with rocking. Finally, the gels were incubated against 2 or 3 changes of 10% acetic acid for 2-3 hours or until the background was clear. When de-staining was complete, the gels were dried on a piece of filter paper as described by Maizel (1971).

D. Preparation of Cell Suspensions and Cell Transfer

1. Spleen cell suspensions

Donor spleen cell suspensions were prepared in RPMI 1640 medium (Grand Island Biological Company (GIBCO), Grand Island, NY) containing 5% fetal calf serum (GIBCO) (RPMI-FCS), by gently teasing the cells with rat-tooth forceps. Cell debris was removed by passing the dissociated cell suspension through a Nitex polyamide nylon screen (Tetko, Inc., Elsford, NY). Dissociated cells were washed once and an aliquot of the cell suspension was counted in a hemocytometer. Viability, as determined by trypan blue exclusion test, was always greater than 90%.

For T-cell depletion of donor cell populations, washed and counted spleen cell pellets were treated with 0.5 ml/spleen of anti-Thy 1.2 (hybridoma HO-13.4), 0.5 ml/spleen of anti-Thy 1 framework (hybridoma T24/40.7) and 1.0 ml/spleen of a 1/5 to 1/10 dilution of rabbit complement (Cedarlane Laboratories Limited, Hornby, Ontario, Canada). The dilution of complement varied from

lot to lot depending on the toxicity of the undilute stock for spleen cells. Dilutions were made with RPMI. Hybridomas HO-13.4 (Marshak-Rothstein et al., 1979) and T24/40.7 (Dennert et al., 1980) were kindly provided by Dr. Karen Elkins, Uniformed Services University of the Health Sciences (USUHS), Bethesda, MD. Treated spleen cells were re-suspended and incubated for 15 minutes at 4°C in an ice bath, then for 30 minutes at 37°C in a water bath placed in a 37°C incubator. Spleen cells were washed twice with RPMI-FCS, re-counted, and checked for viability. The purity of splenic B-cell suspensions obtained by T-cell depletion was ascertained by immunofluorescent staining with fluorescein (Fl) conjugated anti-IgM, Fl anti-IgD and Fl anti-Theta. Cells to be stained were first depleted of erythrocytes by treatment with ammonium chloride lysing buffer (ACK; NIH, Bethesda, MD). T-cell depleted spleen cell pellets were incubated with approximately 1 ml/spleen of ACK for 2 minutes at room temperature. Spleen cells were then washed once with RPMI-FCS. Fluorescent staining and analysis were kindly performed by Ms. Alice B. Davis, NMRI, using a Fluorescence Activated Cell Sorter II (Becton, Dickonson Electronics Laboratory, Mountain View, CA).

Recipients received 10-60 X 10^6 viable untreated spleen cells or 10-25 x 10^6 T-depleted spleen cells intravenously via a lateral tail vein. Generally, pooled

cells from several donors were injected into 3-5 recipients.

2. Peyer's patches suspensions

Donor Peyer's patches were dissected out from the intestine, without removing surrounding tissue or penetrating into the gut lumen, and placed into RPMI-FCS medium. Generally, the small intestine of CBA/Ca mice contained between 10-15 Peyer's patches and that of CBA/N mice, 7-10 Peyer's patches. Single cell suspensions were prepared by passing the cells through a wire screen (60 mesh) into fresh RPMI-FCS medium. The dissociated cells were washed twice and an aliquot of the cell suspension was counted in a hemocytometer. Viability was determined to be about 80% by trypan blue exclusion. Recipients received 5-20 x 10^6 viable Peyer's patches cells intravenously via a lateral tail vein. The donor cell suspension from CBA/Ca mice consisted of Peyer's patches from 19 mice and that from CBA/N mice, consisted of Peyer's patches from 20 mice.

E. Splenic Focus Technique

Recipients received 1500 R of total body irradiation from a cobalt source 4-6 hours before cell transfer. For *in vivo* stimulation studies, recipients were challenged with a stimulating dose (5 x 10^7 colony forming units (CFU)/mouse) of the inactivated preparation of TML intravenously via a lateral tail vein 16 hours after cell transfer. One hour later, spleens were removed and sliced

into 1 mm cube fragments on a McIlwain Tissue Chopper (Brinkman Instruments, Westbury, NY). Fragments were incubated initially for 4-5 hours at 37°C with 95% O_2-5% CO_2 in Complete medium which consisted of Dulbecco's modified Eagle's medium supplemented with 10% agamma horse serum (GIBCO), 50 ng/ml of gentamicin (GIBCO) and 580 ug/ml of L-glutamine (Sigma). The fragments were then individually placed in wells of microtiter plates (Linbro, Flow Laboratories, McLean, VA) and incubated in 0.2 ml per well of Complete medium at 37°C in an atmosphere of 95% O_2-5% CO_2. For in vitro stimulation studies, the fragments were incubated in 0.2 ml per well Complete medium containing various doses of the inactivated TML preparation (see Results section). For either stimulation protocol, culture fluids were removed and replaced with 0.2 ml/well of fresh Complete medium 3 days later and changed at 3-4 day intervals thereafter. Fluids collected 10-23 days after stimulation were assayed for anti-TML antibody, heavy chain class of antibody, and fine specificity of the antibody. Collected culture fluids from individual wells were stored at -20°C until time of assay.

F. Radioimmunoassay (RIA)

1. Immunoadsorbents

Overnight Penassay broth shake cultures of TML were washed twice in PB. The bacteria were suspended to twice the original volume to yield approximately 2 x 10^9 CFU per

ml as determined by cell counts of the final suspension on TSA plates. O.D. 600 readings of overnight Penassay shake cultures of the S. typhimurium rough mutants (described above) were recorded prior to washing these cultures twice in PB. Based on the A_{600} reading obtained for each rough mutant and the previously determined relationship between an A_{600} unit and the number of CFU per ml, the washed bacterial suspensions were adjusted to 2×10^9 CFU per ml in PB. A cell count of the final concentration was also determined on TSA plates. Bacterial lawns of S. pneumoniae were grown on blood agar plates and the organisms were suspended in PB (5 ml/plate). After harvesting, the cultures were washed twice in PB and suspended to one half the original volume. The final concentration yielded approximately 2×10^9 CFU per ml as demonstrated by cell counts on blood agar plates. However, due to autolysis, cell counts were often variable. Therefore, regardless of the cell counts, this procedure was used as standard practice without adjustment of the final volume of the suspension.

Fifty microliters of the appropriate bacterial suspension at approximately 2×10^9 CFU per ml were added to each well of a polyvinyl U-bottom microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA). Wells were allowed to air dry 1-2 days at room temperature. Fixation of the bacteria to the wells was accomplished according to the method of Metcalf and O'Brien (1981) by the addition of

0.2 ml of 0.15% glutaraldehyde in 0.15M PB (pH 7.0). After a 5 minute incubation at room temperature, the glutaraldehyde was removed and the fixation reaction terminated by the addition of 0.2 ml of 0.15M glycine in 0.015M PB (pH 7.0) containing 1% (v/v) agamma horse serum. After fixation, wells were either covered with phosphate-buffered saline (PBS) containing 2.0% horse serum (Biofluids, Inc., Rockville, MD) and stored at 4°C for as long as 2-3 weeks or used immediately in the RIA. The four inactivated preparations of TML described above were also added to microtiter wells (50 ul of a 2 x 10^9 organisms per ml suspension) and used as immunoadsorbents. However, binding of anti-TML antibody in the RIA was not found to be significantly better to any of these inactivated TML immunoadsorbents than binding to the untreated TML immunoadsorbent (data not shown).

TML LPS was re-suspended at a concentration of 2 ug per ml in PB. 50 ul of this suspension was added to wells of a microtiter plate and incubated overnight at 4°C. After removing the LPS, the plates were washed twice with tap water and used immediately in the RIA.

Lipid A was re-suspended to 0.5 mg per ml in distilled water and sonicated for 5 minutes at 75 watts, one time. The suspension was diluted to 10 ug per ml with PB and 100 ul per well were added to microtiter plates. The lipid A-coated plates were incubated overnight at 4°C,

then washed twice with tap water for immediate use in the RIA.

The method used for the adherence of S. typhimurium SH6017 porin protein to microtiter wells was graciously provided by Dr. Jim Douglas, University of Hawaii, Honolulu, HI (unpublished). Briefly, to completely remove traces of SDS from the porin preparation, described above, the protein was precipitated with 6 - 7 volumes of acetone (chilled to -70°C in a dry ice bath) for 1-2 hours at 4°C. The suspension was centrifuged at 10,000 rpm (Sorvall rotor). The acetone phase was removed and replaced with a fresh volume of acetone. After re-suspending the protein pellet, the above precipitation procedure was repeated one time. The pellet was finally re-suspended in 50mM Tris-HC1 (pH 7.0.) A protein determination was performed by the method of Lowry et al. (1951). The porin suspension was adjusted to 5 ug per ml with 0.01M Ammonium Acetate, 0.01M Ammonium Carbonate buffer (pH 8.2). 50 ul per well of the suspension was deposited in microtiter plates. The buffer was evaporated by incubating the porin-coated plates at 37°C, overnight. The plates were washed one time with 0.15% horse serum (HS) in PBS (PBS-HS) and used immediately in the RIA.

The polymerized flagellin preparation was adjusted to 1 ug per ml in PB and 100 ul were added to the wells of microtiter plates. The flagellin-coated plates were incubated overnight at 4°C and washed twice in tap water

immediately before using in the RIA.

2. Procedure

The radioimmunoassay of culture fluids for specific mouse anti-salmonella antibody is a modification of the method originally designed by Segal and Klinman (1976; Metcalf and O'Brien, 1981). Wells containing the appropriate immunoadsorbent were filled with 10% HS in distilled water. Those immunoadsorbents that had been stored in 2% HS were washed one time with PBS-HS before the 10% HS was added. After a minimum incubation period of 30 minutes at room temperature, the plates were washed with PBS-HS. 50 ul of culture fluid from primary B cells, 25 ul of culture fluid from memory B cells, or 25 ul of normal or immune mouse serum were added to each well. All serum dilutions were prepared in 10% horse serum in PBS-HS (PBS-HS-HS). Culture fluids were incubated for 4 hours and serum samples for 2 hours at room temperature. The plates were washed twice with PBS-HS. Upon initial screening for anti-salmonella antibody and fine specificity analysis of TML positive clones, wells were next incubated with 0.1 ml of rabbit anti-mouse (RAM) kappa sera diluted in PBS-HS-HS for 2 hours, followed by overnight incubation at $4^{o}C$ with 100,000 counts per minute (cpm)/well of ^{125}I-labeled guinea pig or goat anti-rabbit (GAR) immunoglobulin sera (see below). The plates were then washed with water, air dried, and individual wells counted in a gamma counter (Searle,

model 1285).

Culture fluids which contained anti-TML antibody were re-analyzed for isotype(s) with the same RIA procedure except that the following RAM class-specific antibodies were used: 1)anti-IgM, 2)anti-IgG3, 3)anti-IgG1, 4)anti-IgG2, 5)anti-IgA.

TML-positive culture fluids were also re-analysed on LPS and S. pneumoniae coated wells. LPS positive clones were re-assayed on rough mutant-coated wells whereas the LPS negative clones were re-assayed on flagellin-, porin-, and lipid A-coated wells.

G. Antisera

The rabbit anti-mouse (RAM) kappa light chain, IgM, IgG2a, and IgG2b sera were purchased from Litton Bionetics, Kensington, MD. RAM IgG3 and IgG1 sera were a gift of Dr. John Cambier (National Jewish Hospital, Denver, CO). All of these antisera were rendered highly specific in our laboratory by absorption on Sepharose columns coupled with myelomas of other isotypes, followed by affinity purification on columns containing myeloma proteins of the appropriate isotype. The resulting antisera were shown to be monospecific (Elkins and Metcalf, 1984) by using anti-PC or anti-levan myeloma and hybridoma antibodies of the IgM, IgG3, IgG2a, IgG2b, IgG1 and IgA classes as described by Liu et al. (1980b). Briefly, 96 well microtiter plates were coated with 100 ul of 1×10^{-4}M solution of PC_{16}-BSA

or of a 500 ug/ml solution of levan from Aerobacter levanicum (Sigma) at 4°C overnight. After washing, the wells were incubated with 10% HS at room temperature for 2 hours. To the washed plates were added 100 ul of protein standard containing 10 ng of the appropriate antigen-specific antibody. The plate was incubated at 4°C for 2-4 hours. After washing, the wells were incubated with 100 ul of a 1 ug/ml dilution of the affinity-purified RAM isotype-specific serum for 2 hours at 4°C. Finally, 100 ul of ^{125}I-GAR containing 100,000 cpm were added to the washed wells and incubated at 4°C overnight. PC_{16}-BSA was prepared in the laboratory of Dr. James J. Kenny, USUHS, as described by Chesebro and Metzger (1972). The PC binding protein standards (IgM, M603; IgG3, F59.6C5; IgG1, 13x.7C9 M511; IgG2b, 103.1C9 T15; IgA, HOPC-8) were kindly provided by Dr. James Kenny. The IgG2a levan-binding protein standard, UPC-10, was purchased from Litton Bionetics. The RAM IgG2b sera was found to be cross-reactive with IgG2a, conversely, the RAM IgG2a sera cross-reacted with IgG2b. Therefore, these two antisera were combined to produce a RAM IgG2 reagent.

Affinity purified RAM IgA sera and immunoadsorbents of purified myeloma proteins, representative of the various isotypes, coupled to cyanogen-bromide-activated Sepharose 4B were kindly supplied by Dr. James Kenny.

H. Iodination

Affinity purified guinea pig anti-rabbit (GAR) immunoglobulin [$F(ab')_2$ preparation] and goat anti-rabbit (GAR) immunoglobulin antisera were prepared by Dr. Fred Finkleman, USUHS. Both GAR antisera were used interchangeably and were iodinated with carrier-free ^{125}Iodine (Amersham Corp., Arlington Heights, IL) by the chloramine T method (Hunter and Greenwood, 1962 ; Klinman and Taylor, 1969).

I. Antigen Binding Standards For RIA

In each RIA, an antigen-specific antibody standard of known binding efficiency was included as a reference for optimal binding conditions. Two anti-S. typhimurium hybridoma antibodies 5D5/IIE12 and 3D9/IID12, generated and affinity purified in our laboratory (Metcalf et al., 1983; Elkins and Metcalf, 1984), have been shown to be specific for the TML LPS and are of the IgG1 and IgG2b isotypes, respectively (Metcalf et al., 1983). Both of these antibodies were used as standards for TML- and TML LPS-specific assays. A 40% ammonium sulfate cut of ascites fluid from BALB/c mice carrying the MOPC-467 myeloma (M467) was the generous gift of Dr. Mason Smith, E. Carolina University School of Medicine, Greenville, NC. This IgA antibody binds to S. typhimurium polymerized flagellin (Smith et al., 1979) and, thus, was used as a standard in flagellin-specific assays and assays which utilize rough

mutants as immunoadsorbents. A rabbit anti-lipid A serum, which was generated according to the procedure of Schuster et al. (1979), was the kind gift of Dr. Mattsby-Baltzer, WRAIR. This antiserum was used as a positive control in lipid A-specific assays.

In all serum assays, a reference immune mouse serum was included. This serum was prepared in our laboratory by the method of Metcalf and O'Brien (1981) by pooling the serum from 50 outbred CD-1 mice that had been immunized 28 days earlier with 50 TML organisms, i.p. This serum was also used as a positive control in porin-specific assays.

A reference anti-S. pneumoniae serum was prepared in our laboratory by immunizing 10 CD-1 mice with 1/100 dilution of Pneumovax Polyvalent pneumococcal vaccine (Merck, Sharp and Dohme Research Laboratories, West Pt., PA) containing 0.5 ug each of 14 pneumococcal capsular types. Serum was collected and pooled 12 days later and included as a positive control on S. pneumoniae-coated plates.

Positive controls for each heavy chain class-specific RAM sera were as follows: 5D5/IIE12, IgG1; 3D9/IID12, IgG2; M467 and CD-1, IgA; H3F1-IF9, IgM; and H3A11-IB6, IgG3. The last two antibody standards, H3F1-IF9 and H3A11-IB6 were culture fluids from two anti-TML hybridomas generated in our laboratory (Elkins and Metcalf, 1984).

J. Statistical Analysis

Statistical analysis was performed using Student's two-tailed t-test for independent means.

III. Results

A. Preliminary Experiments

The splenic focus system is one experimental system which permits an analysis of the B-cell repertoire for a specific antigen. In this system, individual neonatal or adult, primary and secondary clonal precursor B cells can be stimulated (reviewed in Klinman and Press, 1975). *In vitro* fragment cultures, derived from the spleens of irradiated carrier-primed recipients which have received neonatal or adult B cells, permit maximal antigenic stimulation of these donor B cells by providing all ancillary mechanisms of stimulation. Until recently, this system has been used to analyze only those B cells responsive to soluble, chemically defined antigens, such as TNP and PC. However, Gerhard *et al.* (1975) modified the splenic focus system to permit the analysis of the B-cell repertoire specific for a complex natural antigen, influenza virus. However, there have been no studies to date which have analyzed those B cells specific for *Salmonella typhimurium* or any other bacterial organism. To characterize the *S. typhimurium*-specific B-cell repertoire by the splenic focus technique, the parameters of stimulation for this complex antigen had to be determined. The preliminary studies described herein provide the basis for the studies to be described in section B.

1. Form of antigen

S. typhimurium strain TML (TML), a gram-negative bacterium, has a wide array of antigenic determinants. Thus, in order to obtain a physiologically relevant representation of the TML-specific precursor pool, immunization with the viable organism would be optimal. However, unlike synthetic antigens, viable organisms may multiply in fragment cultures leading to contamination of the cultures. Moreover, the dose of the stimulating bacterial inoculum can not be controlled if the organisms are actively multiplying. To circumvent the possibility of contamination, as well as unknown dose effects, the experimental system was developed with an inactivated preparation of the whole organism. Consequently, early log-phase cultures of TML were inactivated by four different methods: shortwave ultraviolet light-irradiation (UV-TML), heat-inactivation (Heat-TML), glutaraldehyde-inactivation (Glut-TML), and acetone killing and drying (AKD-TML).

2. Immunogenicity of inactivated antigen preparations

The four inactivated antigen preparations were compared to determine which antigen preparation would induce the best immune response. The most immunogenic preparation would presumably be able to optimally stimulate most TML-specific B-cell precursors in the splenic focus system.

To fully characterize the B-cell repertoire specific for S. typhimurium and to gain a clearer understanding of the immune mechanisms and the genetics involved in the immune response to this infectious agent, only antibody responses of mice innately resistant to S. typhimurium were selected for this particular study. Those strains which were available for study were the C3H/HeN, AKR, CBA/Ca, and A/J strains of mice. These mice were pre-bled, then immunized intravenously with various doses of each antigen preparation described above. After 28 days, the mice were bled, and their sera assayed for anti-TML antibodies in the solid-phase RIA described in the Materials and Methods. The results of these studies are shown in Figure 2. Panel A demonstrates the response of C3H/HeN mice and Panel B demonstrates the response of AKR mice. In both strains, antigen preparations gave significant anti-TML antibody titers at doses between 1 x 10^5 and 1 x 10^6 organisms injected per mouse. There did not appear to be any significant differences in serum titers between C3H/HeN mice and AKR mice. In addition, none of the antigen preparations could be distinguished as a more effective immunogen by this means. Therefore, serum studies of each preparation in the other mice strains were terminated. However, studies, to be mentioned later, show that the anti-AKD-TML antibody titers in CBA/Ca mice are similar to those of C3H/HeN and AKR mice.

Another approach to evaluate the immunogenicity of

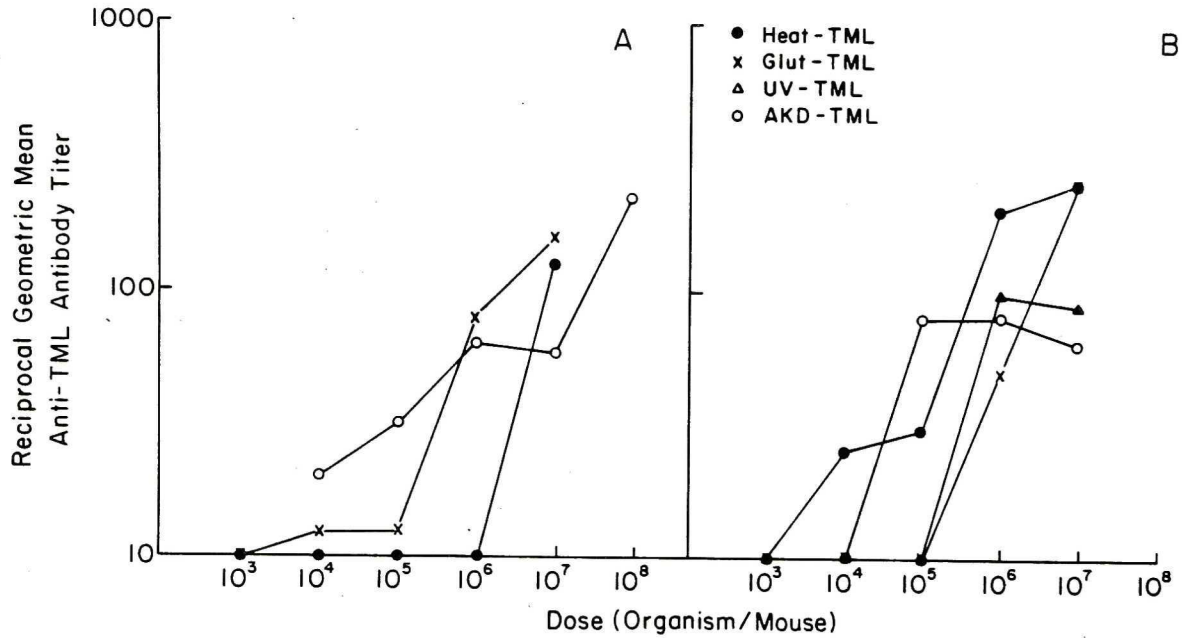

Figure 2. C3H/HeN and AKR mice were pre-bled, then immunized intravenously with various doses of Heat-TML, Glut-TML, or AKD-TML. 28 days after immunization, the serum from each mouse was assayed for anti-TML antibodies by RIA using whole TML organisms as the immunoadsorbent. 2-3 mice were tested at each dose. Each point represents the reciprocal geometric mean anti-TML antibody titer in C3H/HeN (Panel A) and AKR (Panel B) mice.

these antigen preparations would be to determine which preparation could optimally stimulate *in vitro* primary anti-TML antibody responses in unprimed, non-irradiated splenic fragment cultures. This kind of approach would more closely reflect the ability of each inactivated antigen to stimulate TML-specific B-cell precursors in the splenic focus system. However, since B cells would be non-limiting, the culture fluids from individual splenic fragments (derived from unprimed, non-irradiated mice) would presumably contain a heterogeneous mixture of anti-TML antibodies. Braciale *et al.* (1976) used this type of protocol to detect *in vitro* primary influenza virus-specific antibody responses. Although this method could not be used to determine the influenza-specific B-cell precursor frequency nor be used to analyze the antibody product from individual B cells (since B cells were non-limiting), it did permit these investigators to devise a set of parameters necessary to detect individual primary influenza-specific B cells in the splenic focus system. These parameters included the stimulating virus dose and the kinetics of antibody production in splenic fragment cultures. They observed that 9 to 12 days after *in vitro* stimulation of unprimed, non-irradiated fragments with 1,000 hemagalutinin units/ml of influenza virus, 70% of the fragments produced anti-influenza antibody.

To determine the ability of inactivated antigen to

stimulate S. typhimurium-specific B-cell precursors in the splenic focus system, in vitro primary anti-TML antibody responses were obtained using the different antigen preparations that were described previously. These responses were obtained from fragment cultures derived from unprimed, non-irradiated mice which were stimulated either in vitro or in vivo. In the experiment outlined in Table 1, a total of 48 spleen fragments from a pool of 6 C3H/HeN spleens were stimulated in vitro for 3 days at each indicated dose of UV-TML. The culture fluids were assayed by RIA for anti-TML antibody on days 10 and 13 following antigenic stimulation. The frequency of responding fragments, as a percent of total fragments stimulated, was very low at all antigen doses. The largest percentage of fragments responded at the two highest bacterial doses, that is, a mean of 13.5% at 1×10^7 and 1×10^8 organisms/ml. Unstimulated fragments did not produce any anti-TML antibody. Using the same experimental approach, in vitro TNP-Ficoll (TNP-FIC) stimulation yields anti-TNP antibodies in all fragments (E.S. Metcalf, unpublished observation). The small proportion of fragments which produce anti-TML antibody suggests a frequency of TML-responsive B cells much lower than those of TNP-specific and influenza virus-specific B cells. It was not clear whether this low response was due to ineffective presentation of the antigen to the B cells since S. typhimurium was a particulate antigen and might have

TABLE 1

IN VITRO STIMULATION OF ANTI-TML RESPONSE IN UNPRIMED, NON-IRRADIATED SPLENIC FRAGMENT CULTURES[a]

In Vitro Dose of UV-TML/ml	% Responding Fragments[b]
--	0
1×10^4	3.1
1×10^5	2.1
1×10^6	2.1
1×10^7	13.5
1×10^8	13.5

a) A total of 48 spleen fragments from a pool of 6 unprimed, non-irradiated C3H/HeN spleens were stimulated at each antigen dose. UV-TML was added to fragments at the initiation of cultures. Antigen was removed on day 3 and cultures were washed and replaced with fresh culture media. Culture fluids were assayed for anti-TML antibody on days 10 and 13 in a solid phase RIA using whole TML organisms as the immunoadsorbent.

b) The percentage of responding fragments represents the mean number of responding fragments over the total number of fragments tested at a single dose on two consecutive sampling periods (3-day intervals).

difficulties diffusing into the fragment cultures. Microscopic and gross examination of the fragment cultures revealed a layer of bacterial cells settled at the bottom of the microtiter wells. Therefore, to ensure optimal stimulation, all experiments to be described hereafter have been stimulated *in vivo*. The following study examines and determines the optimal *in vivo* stimulating regimen.

Studies by Swanson and O'Brien (1983) demonstrated that intravenously (i.v.) injected TML is cleared from the blood and taken up by the spleen one hour post-inoculation. In addition, studies by E.S. Metcalf (unpublished observation) showed that there was no difference in the TNP-specific precursor frequency detected in the splenic focus system when TNP-FIC was injected 24 hours or 1 hour before recipient spleens were prepared as fragment cultures. Since there appears to be no difference in the TML-specific secondary B-cell response when UV-TML was injected 24 hours or 1 hour before preparing fragment cultures (E.S. Metcalf, unpublished observation), normal non-irradiated C3H/HeN mice were injected i.v. with 1×10^7 UV-TML organisms per mouse at 1/2, 1, 2, and 4 hours before preparation of fragment cultures (Table 2). These results show that 1 hour is sufficient to allow stimulation of TML-specific precursors. There also appears to be no difference in the proportion of fragments producing anti-TML antibody when stimulated either *in vitro* or *in vivo*.

TABLE 2

KINETICS OF IN VIVO STIMULATION OF THE ANTI-TML RESPONSE IN UNPRIMED, NON-IRRADIATED SPLENIC FRAGMENT CULTURES[a]

Post-Injection Period Before Preparation of Splenic Fragments (Hrs.)	% Responding Fragments[b]
1/2	2.1
1	7.3
2	3.1
4	11.5

a) Unprimed, non-irradiated C3H/HeN mice were injected with 1×10^7 UV-TML organisms, i.v., at various times before preparation of splenic fragment cultures. Two mice were tested for each time period. Culture fluids were assayed for anti-TML antibody on day 13.

b) The percentage of responding fragments represents the mean number of responding fragments over the total number of fragments tested at each time period.

After the length of the post-injection period had been determined to be 1 hour, the optimum stimulating dose of each antigen preparation was examined in several strains of mice. Results shown in Table 3 are typical of the responses observed after C3H/HeN, CBA/Ca or A/J mice were stimulated i.v. with various doses of each antigen preparation 1 hour before fragment cultures were prepared. Generally, with increasing antigen dose, there is an increase in the number of responding fragments. The optimum stimulating dose for all antigen preparations in the different strains was usually within the range of 1×10^7 to 1×10^8 organisms per mouse. The decrease in the percent of responding fragments at the highest dose tested indicates that antigen concentrations above 1×10^8 organisms/mouse may be inhibitory to the production of anti-TML antibody-producing foci. This is consistent with the response to DNP-Hy in the splenic focus system (Klinman and Aschinazi, 1971; Klinman, 1972). This Table also shows that peak titers of anti-TML antibody were obtained from culture fluids collected on 13 or 16 days after stimulation. These studies, however, did not indicate which antigen preparation was more immunogenic or a strain of mouse which gave a better anti-TML antibody response. However, these data from unprimed non-irradiated splenic fragment cultures, taken together suggest an extremely low frequency of TML-specific cells in all strains of innately resistant mice tested. As already noted, using a similar

TABLE 3

ANTIGEN DOSE DEPENDENCE OF STIMULATION AND KINETICS OF RESPONSIVENESS OF THE ANTI-TML RESPONSE IN UNPRIMED, NON-IRRADIATED SPLENIC FRAGMENT CULTURES[a]

In Vivo Dose of UV-TML (organisms/mouse, i.v.)	% Responding Fragments at Various Times After Antigen Stimulation[b]		
	day 10	day 13	day 16
1×10^5	0.0	N.D.[c]	1.0
1×10^6	1.0	0.0	0.0
1×10^7	3.6	11.9	14.3
1×10^8	2.1	10.4	7.3

a) Unprimed, non-irradiated C3H/HeN mice were injected with various doses of UV-TML organisms, i.v., one hour before preparation of splenic fragment cultures. Two mice were tested for each dose (approximately 50 fragments/mouse). Culture fluids were assayed for anti-TML antibody at various days after antigen stimulation by solid-phase RIA.

b) The percentage of responding fragments represents the mean number of responding fragments over the total number of fragments tested at each dose for each sampling period.

c) Not done.

protocol, Braciale et al. (1976) were able to detect a much larger frequency of primary influenza virus-specific B cells in fragment culture. They observed that 70% of the fragments responded at the highest stimulating virus concentration. One possible explanation for this apparent dichotomy between the TML-specific and influenza-specific repertoires is that the responsiveness of primary TML-specific B cells may be more T-cell dependent (TD) than primary influenza-specific B cells. Indeed, the low response level observed is similar to that seen when unprimed fragment cultures were stimulated with TD hapten-protein conjugates (see Braciale et al. 1976). The low responses that were detected may be the T-independent (TI) portion of the anti-TML response or possibly a part of the response which may only need unprimed T cells or viable macrophages. Nevertheless, these studies have supplied a framework with which to initiate the limiting dilution cell transfer system.

3. Variables associated with cell transfers

Recent studies by O'Brien and Metcalf (1982) demonstrated that the salmonella-resistance conferred by the Ity^r allele does not require functional T lymphocytes, since the kinetics of bacterial growth in the spleens and livers of euthymic nu/+ and athymic nu/nu mice of the Ity^r phenotype were similar during the early phase of infection. In contrast, other studies by these investigators (O'Brien

et al., 1981) suggested that the anti-salmonella antibody response is TD since Ity^r nude mice die late in the course of the infection. Furthermore, these nude mice produced only low levels of IgM anti-TML antibody with no detectable levels of IgG antibody after vaccination with AKD-TML. Since the studies described in the preceding section support the suggestion that the antibody response to S. typhimurium is TD, it was decided that the TML-specific B-cell precursor pool could be most effectively analyzed in the splenic focus system using irradiated, TML-antigen-primed recipients which could serve as a source of TML-specific helper T cells and macrophages. Previous studies by Pierce and Klinman (1977) demonstrated that carrier-specific enhancement could be maximized for B cells from non-immunized donors when transferred to immunized recipients which are syngeneic at the H-2 locus but allogeneic at the immunoglobulin heavy chain (IgCH) genes. This donor-recipient combination apparently circumvents an antibody-specific immunoregulatory mechanism present in immunized recipients which suppresses stimulation of syngeneic, primary B cells. Indeed, this was found to be the situation in the influenza hemagglutinin (HA) system. Cancro et al. (1977) found that when B10.D2 recipients, primed with the PR8 strain of influenza, received syngeneic donor B cells they could detect only a very few PR8 HA-specific precursor cells. However, when PR8-primed B10.D2

recipients received BALB/c donor B cells (H-2 syngeneic, but allotypically different at the IgCH locus), the primary PR8 HA-specific precursor response was enhanced several-fold. This type of phenomenon was never observed in primary responses to simple haptenic determinants since it was possible to prime recipients with the carrier molecule alone. Thus, recipients were not exposed to the haptenic determinant and hence, did not develop a regulatory mechanism which would suppress syngeneic hapten-specific B-cell responses. On the other hand, it is not possible to obtain carrier-specific help for primary responses to complex antigens, like PR8, by immunizing with the whole virus. Such an immunogen contains both carrier determinants and haptenic determinants. Therefore, carrier-specific helper T cells as well as hapten-specific (HA-specific) B cells were primed when recipients were immunized with PR8 virus. At the same time, however, a regulatory mechanism was apparently induced in the recipients which would suppress primary syngeneic B cells responsive to the priming antigen. As a result primary HA-specific B cells transferred to these PR8-primed syngeneic recipients would be suppressed. Pierce and Klinman (1977) showed that this regulatory circuit could be circumvented using the H-2 identical, allotype distinct transfer system.

Since recipients were to be immunized with whole [inactivated] TML organisms, it was probable that primary B cells specific for TML would be suppressed when transferred

to syngeneic recipients. Moreover, since the above preliminary studies suggested that the frequency of TML-specific B cells was low, it was possible that the donor-recipient strain combination described by Pierce and Klinman could amplify responsiveness to TML. Strains of mice available in appropriate combinations are listed in Table 4. B cells from salmonella-resistant, immunologically normal CBA/Ca mice were selected as the strain of mouse in which to study the TML-specific repertoire. This strain was chosen, in part, because a mutant strain of CBA/Ca mice was also available which was susceptible to S. typhimurium (O'Brien et al., 1979b). Thus, in addition to trying to understand the repertoire in normal mice, it would be possible to compare the repertoire in normal mice to that in susceptible mice. The analysis of the B-cell responses in these strains of mice would also provide insights on the role of B cells in resistance to murine typhoid and on the genetics and immune mechanisms responsible for the differential susceptibility of mice to this bacterial infectious disease agent. Consequently, the recipient strain could either be the AKR strain or the B10.BR strain (Table 4). Unlike the AKR strain, B10.BR mice are innately susceptible to S. typhimurium since they carry the homozygous recessive allele for Ity (Ity^s). As will be shown in later experiments, the Ity^s phenotype apparently has no effect on the ability to provide T-cell

TABLE 4

READILY AVAILABLE H-2 IDENTICAL,

ALLOTYPICALLY DIFFERENT MOUSE STRAINS

Strain	H-2 Haplotype[a]	IgCH Allotype[b]
CBA/Ca	k	j
C3H/HeN	k	j
AKR	k	d
B10.BR	k	b

a) (Staats, 1972)

b) (Lieberman, 1978)

and macrophage help for stimulation of TML-specific B cells in this system. Therefore, both AKR and B10.BR mice were used as recipients throughout the studies described in this thesis.

Once the donor strain had been selected, it was necessary to ensure that the splenic focus system allowed the analysis of the antibody product of a single TML-specific B cell. Therefore, varying numbers of non-immune CBA/Ca donor spleen cells were injected into 2-3 lethally irradiated, AKD-TML primed, AKR recipients. All recipients were subsequently challenged with 5×10^7 AKD-TML organisms. The data in Figure 3 are plotted both as the average number of detected anti-TML antibody-producing fragments per recipient spleen and as the estimated number of TML-specific precursor B cells per recipient spleen calculated by the Poisson formula, $P(x)= \frac{e^{-u}u^x}{x!}$. The estimate of the average number of stimulatable precursor cells per fragment (u) was based on the fraction of non-responding fragments, $P(0)=e^{-u}$, since the negative logarithm of the fraction of non-responding fragments is linearly proportional to the mean number of precursor cells per fragment. The number of responding fragments followed a Poisson distribution and a single limiting cell type is indicated from the one to one linear relationship between the number of responding fragments and the number of cells injected. Forty $\times 10^6$ spleen cells per recipient has

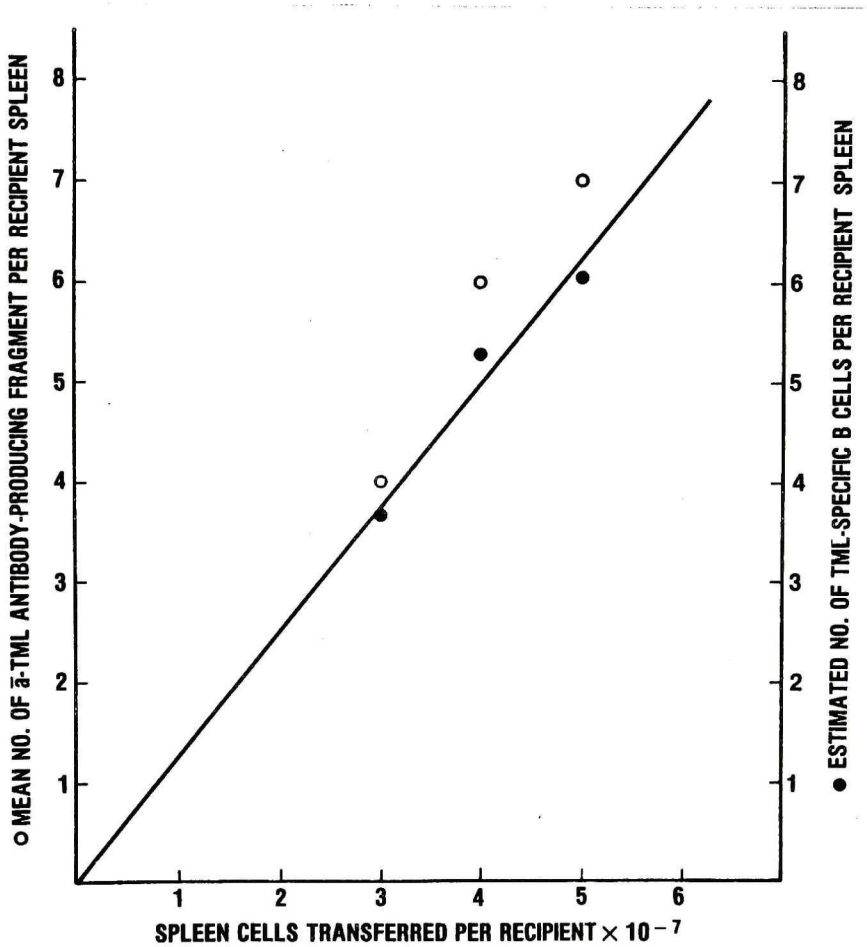

Figure 3. 30-50 x 10^6 unprimed CBA/Ca donor spleen cells were injected into AKD-TML primed AKR recipients. Priming and stimulation of recipients and detection of clones by RIA were as described in Figure 7. The data are plotted both as the average number of detected anti-TML antibody-producing fragments per recipient spleen and as the estimated number of TML-specific precursor B cells per recipient spleen calculated by Poisson analysis. The straight line was fitted through the origin by the method of least squares.

reproducibly yielded splenic fragment cultures which produce TML-specific monoclonal antibody and, henceforth, this number was selected as the optimum number of cells to be transferred.

4. Antigen dose dependence and kinetics of priming

To ensure maximum B-cell stimulation in recipient fragment cultures, it was necessary to generate optimal ancillary mechanisms in recipient mice. Generation of such mechanisms depends upon an immunogenic antigen and on an immunization regimen which most effectively stimulates and expands antigen-specific helper T cells and macrophages. To determine which antigen preparation would optimally serve this purpose, the same AKR mice which had been immunized with the various doses of each antigen preparation for the serum studies described above (Figure 2), were later used as primed recipients in the splenic focus system. Five to 7 weeks after immunization, these AKR recipients were irradiated, injected with 20×10^6 CBA/Ca or C3H/HeN donor spleen cells and subsequently challenged with approximately 5×10^7 organisms per mouse of the same antigen preparation used for priming. The results (Figure 4) are shown both as the serum anti-TML antibody titer one week before cell transfer and as the number of antibody-producing foci per 10^6 spleen cells transferred (frequency). Detectable frequencies were observed when 1×10^3 and 1×10^4 organisms/mouse of each

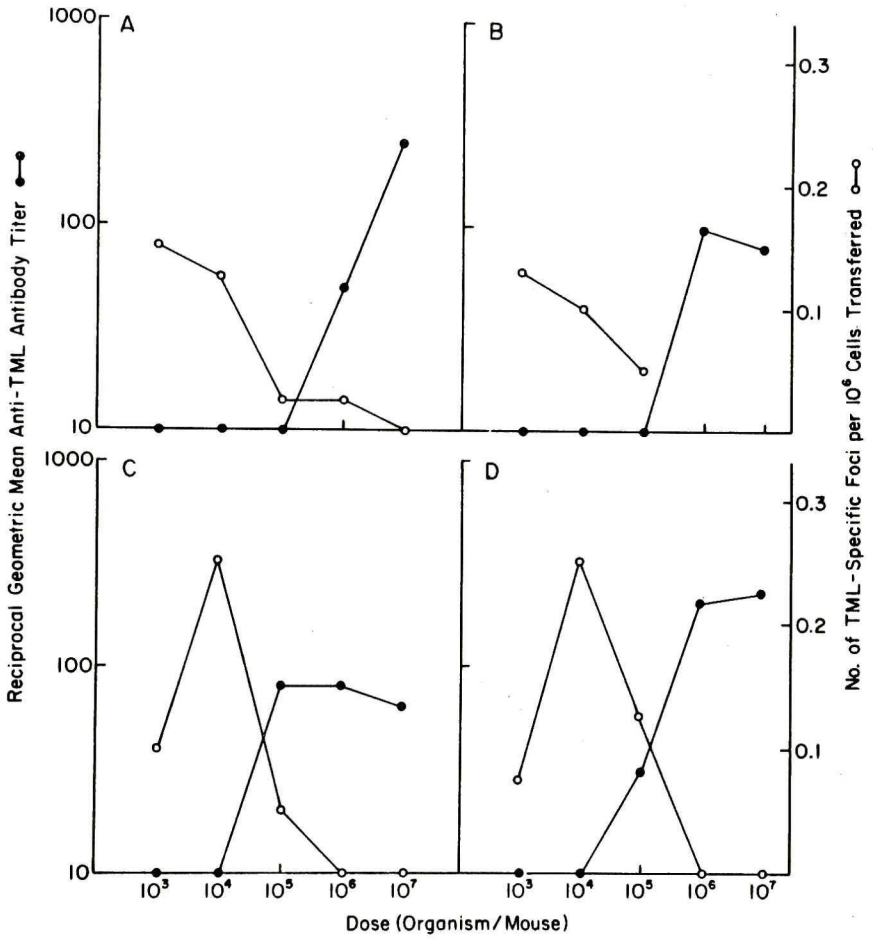

Figure 4. AKR mice were pre-bled, then immunized intravenously with various doses of each antigen preparation. Five to 7 weeks after immunization, these AKR mice were used as primed recipients in the splenic focus system. One week before cell transfer, the serum from each primed recipient was assayed for anti-TML antibody by RIA using whole TML organisms as the immunoadsorbent. Two to 3 recipients were tested at each priming dose. Antigens used for recipient priming and stimulation were as follows: Panel A, Glut-TML; Panel B, UV-TML; Panel C, AKD-TML; Panel D, Heat-TML.

antigen preparation were used to prime recipients. Therefore, low doses of all of these antigens were apparently able to prime helper T cells, but these doses were insufficient to stimulate serum antibody responses in these recipients. As priming doses were increased above 1 x 10^4 organisms/mouse, recipient serum titers increased with a parallel increase in antibody secreted from primed recipient fragment cultures that had received no donor cells. As a consequence, at high priming doses it was difficult to ascertain whether the antibody detected in the fragment culture supernatants was derived from the transferred spleen cells or the recipient fragment cultures. Thus, the backgrounds were high enough to mask specific responses. The decrease in frequency to background levels, shown in Figure 4, reflects this difficulty. Additional comparative studies suggested that 1 x 10^3 AKD-TML organisms/mouse was the optimum dose and antigen preparation for inducing the highest frequency of TML-specific precursors (data not shown) and was used for all further studies.

Initially, the AKR strain of mice was chosen as the recipient of choice because this strain is innately resistant to S. typhimurium. However, upon closer examination of the antigenic specificities of the immunoglobulin allotypes (Table 5), it was discovered that there were fewer differences between the allotypes of

TABLE 5

COMPARISON OF ANTIGENIC SPECIFICITIES

BETWEEN ALLOTYPICALLY DIFFERENT STRAINS OF MICE[a]

Class	Antigenic Specificity in CBA/Ca Mice	No. of Specificities Shared with CBA/Ca by: AKR/J	B10.BR
IgG2a	1, 6, 7, 8, 28, 29, 30	4	1
IgG2b	9, 11, 22, 31, 33, 34	2	4
IgG1	8, 19	2	0
IgA	12, 13, 14	1	0
IgM	N.T.[b]	N.T.	
IgD	36	1	0

a) (Liebermann 1978)

b) Not tested.

CBA/Ca and AKR mice than between CBA/Ca and B10.BR mice. These mice, as stated earlier, are susceptible to S. typhimurium due to their expression of the Ity^s allele. However, since it has been shown that functional T cells are not required for the expression of salmonella-resistance conferred by the Ity^r allele (O'Brien and Metcalf 1982), it seemed likely that B10.BR mice would be able to provide helper T cells which were normal. Furthermore, greater disparity at the IgCH locus between donors and recipients could permit a greater number of TML-specific precursor B cells to be stimulated. It was possible that the antibody-specific immunoregulatory mechanism discussed above, could have suppressed some of the CBA/Ca primary TML-specific B cells which share common antigenic specificities with allotype markers in AKR mice. Therefore, the frequencies of TML-specific B cells in both AKR and B10.BR were compared. 40×10^6 CBA/Ca spleen cells were injected into either AKR or B10.BR recipients previously primed with 1×10^3 AKD-TML organisms 4 weeks before cell transfer. The frequency data in Table 6 shows that under these conditions, B10.BR mice provide a better environment for stimulation of TML-specific B cells than do AKR mice. However, Table 7 shows that if AKR mice are given booster immunizations, the frequency of TML-specific cells increases. By immunizing AKR recipients with 1×10^3 AKD-TML organisms/mouse i.v. 14 days, 11 days, and 7 days before cell transfer (Table 7, line 7), the frequency is

TABLE 6

EFFECT OF DIFFERENT RECIPIENTS AS SOURCES OF T CELLS AND MACROPHAGES ON THE FREQUENCY OF PRIMARY TML-SPECIFIC B CELLS[a]

Total Donor Cells Transferred ($\times 10^6$)	Recipient	No. TML-specific Clones per 10^6 Spleen Cells Transferred[b]
280	AKR/J	0.086 $\pm$ .008
200	B10.BR	0.205 $\pm$ .008

a) In each experiment, 40 x 10^6 CBA/Ca donor spleen cells were injected into recipients previously primed with 1 x 10^3 AKD-TML organisms, i.v., 4 weeks before cell transfer. Stimulation of recipients and detection of clones by RIA were as described in Figure 7.

b) Mean frequency $\pm$ 2 standard errors. As assessed by Student's t-test, the frequency of TML-specific clones was significantly ($p<0.05$) higher in B10.BR fragment cultures than in AKR fragment cultures.

TABLE 7

EFFECT OF DIFFERENT PRIMING REGIMENS IN AKR MICE ON THE FREQUENCY OF PRIMARY TML-SPECIFIC B CELLS[a]

Time of Priming Before Cell Transfer	No. TML-specific Clones per 10^6 Spleen Cells Transferred[a]
3 days	0.060 ± 0.043[b]
7 days	0.128 ± 0.020
10 days	0.113 ± 0.018
14 days	0.072 ± 0.010
28 days	0.096 ± 0.015
10 days + 7 days	0.117 ± 0.026
14 days + 11 days + 7 days	0.208 ± 0.059
4 weeks + 2 weeks	0.188 ± 0.053
6 weeks + 2 weeks	0.050 ± 0.017
6 weeks + 4 weeks + 2 weeks[c]	0.092 ± 0.051

a) In each experiment 40 x 10^6 CBA/Ca donor spleen cells were injected into AKR/J recipients which had been previously primed with 1 x 10^3 AKD-TML organisms, i.v., at various times before cell transfer. Each experiment had two to six primed recipients. Stimulation of recipients and detection of clones by RIA were as described in Figure 7.

b) Mean frequency ± 2 standard errors.

c) Recipients primed with 1 x 10^3 AKD organisms in CFA, i.p., 6 weeks before cell transfer, then with 1 x 10^3 AKD organisms in saline, i.v., 4 weeks and 2 weeks before cell transfer.

increased to the level observed in B10.BR recipients primed one time, 4 weeks before cell transfer. In addition, when AKR recipients are primed by a standard regimen used for detecting responses to synthetic antigens, that is, by immunizing with 1 x 10^3 AKD-TML emulsified in complete Freund's adjuvant (CFA) 6 weeks prior to cell transfer followed by 2 booster injections with AKD-TML in saline 4 weeks and 2 weeks before (Table 7, line 10), the frequency of TML-specific B cells is equivalent to the frequency in AKR recipients primed with one dose 4 weeks before cell transfer. However, it should be noted that this frequency is not significantly different ($p>0.10$) from that obtained in AKR recipients primed 14 days, 11 days, and 7 days before cell transfer. As Table 7 shows, several different priming regimens were examined. Though the 14 day, 11 day, and 7 day sequence of immunizations did not generate significantly higher ($p>0.05$) frequencies than the other multiple immunization sequences, the values obtained from recipients primed by this regimen were consistently greater than those obtained by any other immunization protocol. Hence, the 14 day, 11 day, and 7 day sequence was empirically the most effective regimen for AKR mice. Furthermore, this sequence of immunizations generated TML-specific precursor frequencies which were significantly higher ($p<0.05$) than those generated in recipients primed by most of the single immunization regimens, i.e. the 3

day, the 14 day, and the 28 day regimens.

The optimum priming regimen for AKR mice appeared to be inhibitory for responsiveness in B10.BR primed fragments (Table 8, line 3). The peak response in B10.BR primed recipients was detected after only one immunization with AKD-TML between 3 and 6 weeks before cell transfer. Thus, by using the appropriate priming regimen, both AKR and B10.BR mice appear to generate equivalent levels of TML-primed helper T cells.

Although the *in vivo* immune response to *S. typhimurium* late in the infection appears to be TD (O'Brien *et al.*, 1981; O'Brien and Metcalf, 1982) and though the results observed thus far in this thesis support this contention, the possibility that the response to AKD-TML in the splenic focus system is TI has not been ruled out. Therefore, to determine the T-dependency of the anti-TML antibody response, 34-40 x 10^6 CBA/Ca donor spleen cells were transferred to unprimed AKR or B10.BR recipients. As shown in Table 9, TML-specific precursors were detectable only in recipients which have been previously primed with AKD-TML since the frequency of clones obtained from unprimed recipients (Table 9) was equivalent to the number obtained from primed recipient controls which did not receive any donor spleen cells (data not shown). These results suggest that primary anti-TML antibody responses are exquisitely dependent on antigen-primed recipient T cells in this experimental system.

TABLE 8

EFFECT OF DIFFERENT PRIMING REGIMENS IN B10.BR RECIPIENTS ON THE FREQUENCY OF PRIMARY TML-SPECIFIC B CELLS[a]

Time of Priming Before Cell Transfer	No. TML-specific Clones per 10^6 Spleen Cells Transferred[b]
7 days	0.056 ± 0.021
10 days + 7 days	0.044 ± 0.012
14 days + 11 days + 7 days	0.075 ± 0.017
3 weeks	0.156 ± 0.026

a) 40×10^6 CBA/Ca donor spleen cells were injected into B10.BR recipients which had been previously primed with 1×10^3 AKD-TML organisms, i.v., at various times after cell transfer. Stimulation of recipients and detection of clones by RIA were as described in Figure 7.

b) Mean frequency ± 2 standard errors.

TABLE 9

FREQUENCY OF PRIMARY TML-SPECIFIC B CELLS DERIVED FROM UNPRIMED AND PRIMED RECIPIENT FRAGMENTS[a]

Total No. of Cells Analyzed ($\times 10^6$)	Recipient	No. TML-Specific Clones per 10^6 Spleen Cells Transferred[b]
342	unprimed	0.015 ± 0.005
1090	AKD-TML primed	0.154 ± 0.009

a) $30\text{-}50 \times 10^6$ CBA/Ca donor spleen cells were injected into either unprimed AKR/J and B10.BR recipients or into AKR/J and B10.BR recipients which had been previously primed with 1×10^3 AKD-TML as described in Figure 7.

b) Mean frequency $\pm$ 2 standard errors. As assessed by Student's t-test, the frequency of TML-specific clones was significantly ($p<0.05$) higher in primed recipient fragment cultures than in unprimed fragment cultures.

A final factor which may influence the generation of maximal helper T cell and macrophage activity in recipients is the route of immunization. The i.v. route has been criticized as not being a physiologically relevant route of inoculation (Herzberg et al., 1972). The i.p. route, on the other hand, has been considered to more closely resemble the natural infection since organisms pass through lymphoid barriers before entering the circulation from the peritoneum when injected by this route. In contrast, organisms introduced i.v. are lodged in the liver and spleen without prior involvement of the lymphatic system (Collins, 1969; Herzberg et al., 1972). Therefore, priming by the i.p. route may enhance local, regional, and systemic immune mechanisms. However, i.v. injection may provide greater stimulation of the immune system since the organisms may readily be disseminated throughout the RES by this systemic route. Therefore, the detection of TML-specific B cells in fragment cultures derived from recipients primed by different routes was examined. AKR mice were immunized i.v. or i.p. with 1 x 10^3 AKD-TML organisms 5 weeks before transfer of unprimed CBA/Ca spleen cells. Table 10 shows that there is no difference ($p>0.4$) between the frequency of TML-specific cells stimulated in recipients primed i.v. and those primed i.p. This finding was expected since studies by Metcalf and O'Brien (1981) demonstrated that the kinetics and titers of the serum

Table 10

EFFECT OF DIFFERENT ROUTES OF RECIPIENT PRIMING ON THE FREQUENCY OF PRIMARY TML-SPECIFIC B CELLS[a]

Route of Priming	Total No. of Cells Transferred (x 10^6)	No. of TML-Specific Clones per 10^6 Spleen Cells Transferred[b]
Intravenous (i.v.)	60	0.10 ± 0.03
Intraperitoneal (i.p.)	60	0.15 ± 0.09

a) 20 x 10^6 CBA/Ca donor spleen cells were injected into AKR/J recipients which had been previously primed with 1 x 10^3 AKD-TML organisms by either the i.v. or i.p. route 5 weeks before cell transfer.

b) Mean frequency ± 2 standard errors. As assessed by Student's t-test, there is no significant ($p>0.4$) difference between the frequency of TML-specific clones stimulated in recipients primed i.v. and those primed i.p.

anti-TML antibody responses were similar after either i.v. or i.p. challenge with a low dose of live organisms. However, the initial rise in both IgM and IgG titers (2 and 3 weeks post-inoculation, respectively) occurred 1 week earlier in mice immunized by the i.v. route, presumably because the direct injection into the bloodstream allows the organisms to reach the spleen more quickly than by the i.p. route. The results presented by Metcalf and O'Brien are consistent with the kinetics of growth of S. enteritidis in the blood, liver, and spleens of mice challenged by the i.v. and i.p. routes (Collins, 1969). Therefore, although the study presented in Table 10 uses recipients primed 5 weeks before cell transfer, it is more than likely that the 14 day, triple i.v. immunization protocol described above (Table 7) will be just as, if not more effective than, an i.p. immunization route.

5. Antigen dose dependence of stimulation

The studies described in section A.2. which detected anti-TML antibody responses in fragment cultures derived from unprimed, non-irradiated recipients, indicated that antigen doses between 1×10^7 and 1×10^8 organisms yielded the greatest percentage of responding fragments (Table 3). Figure 5 shows the relationship between the frequency of TML-specific B cells and the antigen concentration used for stimulation. It can be seen, although there are no significant differences in this

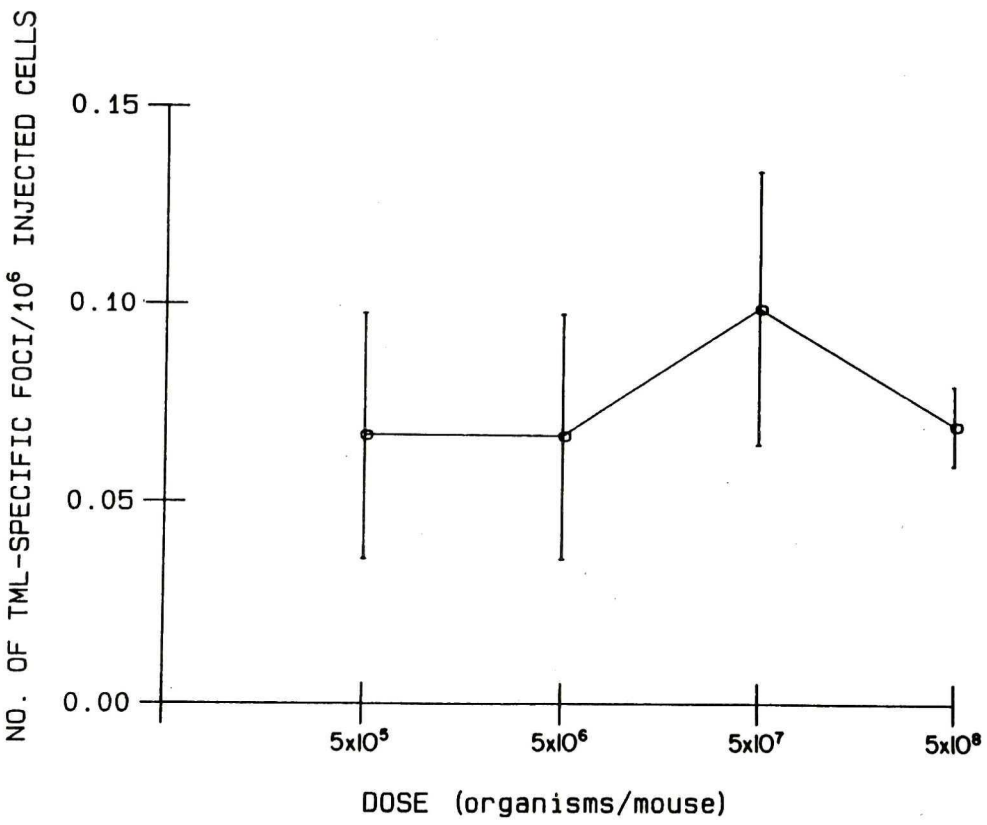

Figure 5. Relationship between the frequency of TML-specific B cells and the antigen concentration used for stimulation. 25 x 10^6 unprimed CBA/Ca donor spleen cells were injected into B10.BR recipients which had been previously primed as described in Figure 7. Recipients were subsequently injected with varying doses of AKD-TML. Culture fluids were assayed for anti-TML antibody by RIA using whole TML organisms as the immunoadsorbent. Each point represents the mean frequency $\pm$ 2 standard errors. As assessed by Student's t-test, stimulation with 5 x 10^7 AKD-TML organisms does not generate significantly greater numbers of foci than do the other stimulating doses ($p>0.4$).

study, that stimulation with 5×10^7 organisms appears to produce the greatest number of detectable foci. Furthermore, the amount of antibody produced per focus was greater at this antigen concentration than at other concentrations (data not shown). Therefore, 5×10^7 AKD-TML organisms was selected as the antigen concentration of choice for the following studies.

6. Kinetics of responsiveness

Culture fluids from fragments containing anti-TML antibody-producing foci contained undetectable levels of antibody on the seventh day of culture. By the tenth day after stimulation, these culture fluids began to show measurable antibody (Figure 6). Maximum antibody was released by day 16 in culture. Antibody production usually continued for 24 days after fragment cultures were established. Comparable kinetics have been observed for the primary HA-specific response in the splenic focus system (Cancro et al., 1978; Wylie and Klinman, 1981) Contrary to the responses of these natural complex antigens, antibody to synthetic hapten-carrier conjugates are first detected in culture fluids collected at days 6 to 8 and reach a maximum by day 12 in culture (Klinman and Aschinazi, 1971; Klinman, 1972).

Collectively, the preliminary studies presented in this section define the experimental procedures (diagrammed in Figure 7) required to maximize monoclonal responses to

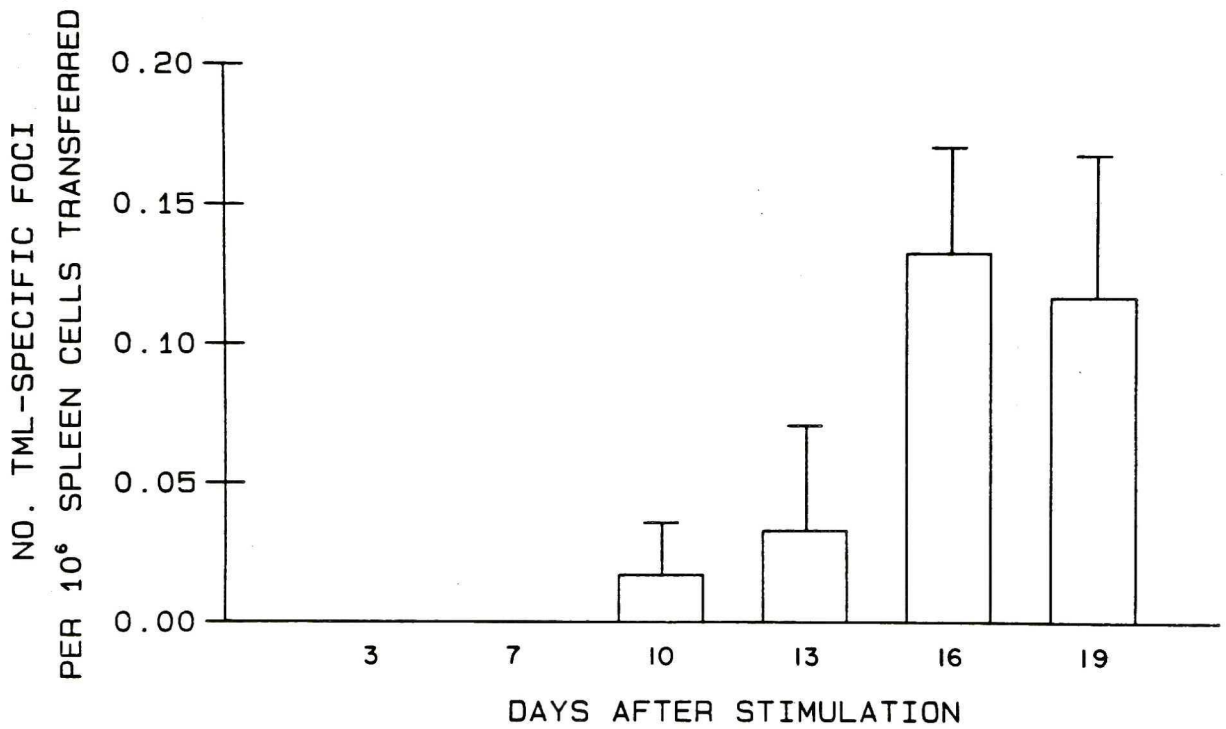

Figure 6. Kinetics of primary TML-specific B-cell responsiveness in splenic fragment cultures. 20 x 10^6 CBA/Ca donor spleen cells were injected into AKR recipients which had been primed 4 weeks earlier with 1 x 10^3 AKD-TML organisms. Culture fluids from fragment cultures were assayed on various days after antigen stimulation by RIA using whole TML organisms as the immunoadsorbent. The data are presented as the mean frequency $\pm$ 2 standard errors.

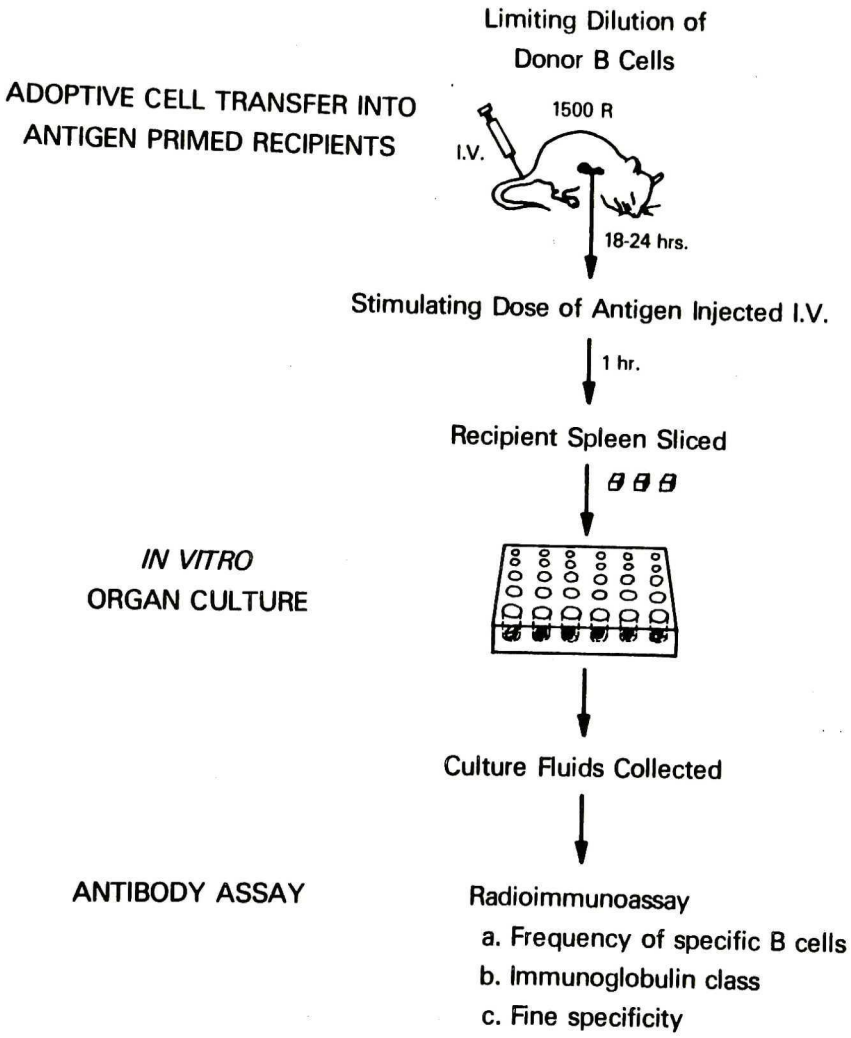

Figure 7. Splenic focus system modified for analyzing Salmonella typhimurium-specific B cells. Generally, 40 x 10^6 unprimed CBA/Ca donor spleen cells are injected into primed AKR or B10.BR recipients. AKR recipients are primed with 1 x 10^3 AKD-TML organisms i.v. 14 days, 11 days, and 7 days before cell transfer. B10.BR recipients are primed 3 weeks before cell transfer with 1 x 10^3 AKD-TML i.v. Primed recipients are stimulated with 5 x 10^7 AKD-TML organisms. Culture fluids are assayed for anti-TML antibody 16, 19, and 22 days after antigen stimulation in a solid-phase RIA.

AKD-TML by non-immune CBA/Ca B cells in primed recipient, splenic fragment cultures. It is now possible to study individual S. typhimurium-specific B cells and their antibody products.

B. Analysis of the Primary TML-Specific B-Cell Response in CBA/Ca Mice

1. Frequency of splenic primary TML-specific B cells

To measure the frequency of primary clonal precursor cells specific for TML in salmonella-resistant, immunologically normal mice, 30-50 x 10^6 non-immune CBA/Ca spleen cells were injected either into lethally irradiated (1500 R) AKR/J recipients which had been primed i.v. 14 days, 11 days, and 7 days before with 1 x 10^3 AKD-TML organisms/mouse or into lethally irradiated B10.BR recipients which had been primed one time i.v. with the same dose of AKD-TML 3 weeks before. All recipients were subsequently stimulated with 5 x 10^7 AKD-TML organisms/mouse one hour before preparation of fragment cultures. The results obtained from several experiments using both AKR and B10.BR recipients are presented in Table 11. The frequency data, expressed directly as the number of B-cell precursors per 10^6 B cells, were calculated using the donor cell cloning efficiency and the percentage of B cells in the CBA/Ca spleen. The donor cell cloning efficiency was previously determined for the splenic focus system to be 4.0% (Klinman et al., 1976), and the percentage of B cells in CBA/Ca spleen has been determined to be approximately 40% (Scher et al., 1975). The results show that the frequency of TML-specific B cells is quite low compared to the frequencies observed for several

TABLE 11

FREQUENCY OF PRIMARY *S. TYPHIMURIUM*-SPECIFIC

B CELLS IN ADULT CBA/Ca MICE

Experiment	Total Donor Cells Transferred (x 10^6)	Recipient (AKD-TML Primed)	No. TML-Specific Clones Per 10^6 Spleen Cells Transferred[a]	No. TML-Specific Cells Per 10^6 Splenic B Cells[b]
1	120	AKR/J	0.21 ± 0.06	13.0 ± 3.7
2	330	AKR/J	0.14 ± 0.01	8.8 ± 0.6
3	120	AKR/J	0.10 ± 0.02	6.3 ± 1.1
4	120	B10.BR	0.22 ± 0.01	13.6 ± 0.6
5	120	B10.BR	0.16 ± 0.04	9.9 ± 2.4
6	160	B10.BR	0.16 ± 0.03	9.8 ± 1.6
7	120	B10.BR	0.12 ± 0.05	7.5 ± 2.9

a) 30-50 x 10^6 CBA/Ca donor spleen cells were injected into AKD-TML primed recipients. Each experiment had two to eight primed recipients. Priming and stimulation of recipients and detection of clones by RIA were as described in text and Figure 7. Data are presented as mean frequency ± 2 standard errors. Mean frequency for experiments 1-3 = 0.15 ± 0.01 and for experiments 4-7 = 0.16 ± 0.09.

b) Calculated frequencies after homing efficiency and percent of B cells in the spleen were taken into account (Klinman *et al.*, 1976). Mean frequency for experiments 1-3 = 9.1 ± 0.5 and for experiments 4-7 = 10.1 ± 0.6.

haptenic determinants (see Sigal and Klinman, 1978), with no apparent difference between the mean number of precursors detected in AKR-derived fragment cultures and the mean number detected in B10.BR-derived fragment cultures (9.1 per 10^6 B cells and 10.1 per 10^6 B cells, respectively).

The observed frequency of 9-10 TML-specific cells per 10^6 B cells is 15-20 fold lower than the frequencies obtained with many haptenic determinants, (see Sigal and Klinman 1978); however, this frequency is similar to the frequency observed for the natural complex antigen influenza HA (13 per 10^6 B cells; Cancro et al., 1978) and to that of the haptenic determinent phosphorylcholine (PC), which is found naturally in the cell wall of Streptococcus pneumoniae (19 per 10^6 B cells; Sigal et al., 1975).

2. Isotype analysis of monoclonal anti-TML antibodies

The isotype of the antibody produced by TML-specific clones obtained in the above experiments was determined by RIA using heavy chain class-specific rabbit anti-mouse immunoglobulin sera. The clones were tested using whole TML as an immunoadsorbent and usually represented a pool of day 16, 19, and 22 culture fluids. Thus, the pooled culture fluids contained the antibody produced by a single TML-specific clone during a 9-10 day period. As a consequence, monoclonal antibody from such a pool may contain several isotypes due to class-switching

events which may have occurred during this period of time (Gearhart et al., 1975b, 1980; Teale et al., 1981; Mongini et al., 1982, 1983). The percent of primary TML-specific clones which secreted a particular isotype is shown in Table 12. The results indicate that the predominant isotype produced is IgM followed by equivalent, but three-fold lower, levels of IgG2 and IgA. The IgG3 and IgG1 isotypes were much less frequently produced. Previous studies (Press and Klinman, 1973c, Slack et al., 1980) have shown that the predominant IgG subclass stimulated by TD antigens is IgG1. On the other hand, type 1 TI (TI-1) antigens were found to stimulate similar levels of IgG3 and IgG2 subclasses, and type 2 TI (TI-2) antigens were found to predominantly stimulate the IgG3 subclass (Slack et al., 1980). Figure 8 compares the relative frequency of heavy chain isotypes produced by primary TML-specific clones to the frequencies produced by clones from B cells responsive to the TD antigen, DNP-Hy, and to the TI-2 antigen TNP-FIC. Although significant and similar amounts of IgA are stimulated by both TML and TNP-FIC, this isotype, together with IgM, dominate the response to the TI-2 antigen. In contrast, the occurrence of IgA in the TML-specific response is 3-fold lower than the occurrence of IgM. Unlike the responses of either TI-1 or TI-2 antigens, the majority of the IgG antibody response to TML is IgG2. It is possible that the priming regimen used in these studies does not optimally stimulate helper T cells. This could

TABLE 12

PROPORTION OF CLONES FROM PRIMARY TML-SPECIFIC CELLS WHICH SECRETE A PARTICULAR ISOTYPE

No. Clones Analyzed[a]	% of Clones Secreting:[b]				
	IgM	IgG3	IgG1	IgG2	IgA
158	75.3	9.5	3.8	22.2	23.4

a) TML-specific clones from unprimed CBA/Ca spleen cells were obtained from fragment cultures derived from AKD-TML primed AKR or B10.BR recipients.

b) The isotype of the antibody produced by primary TML-specific clones was determined by RIA using whole TML organisms as the immunoadsorbent and rabbit anti-mouse heavy chain-specific sera.

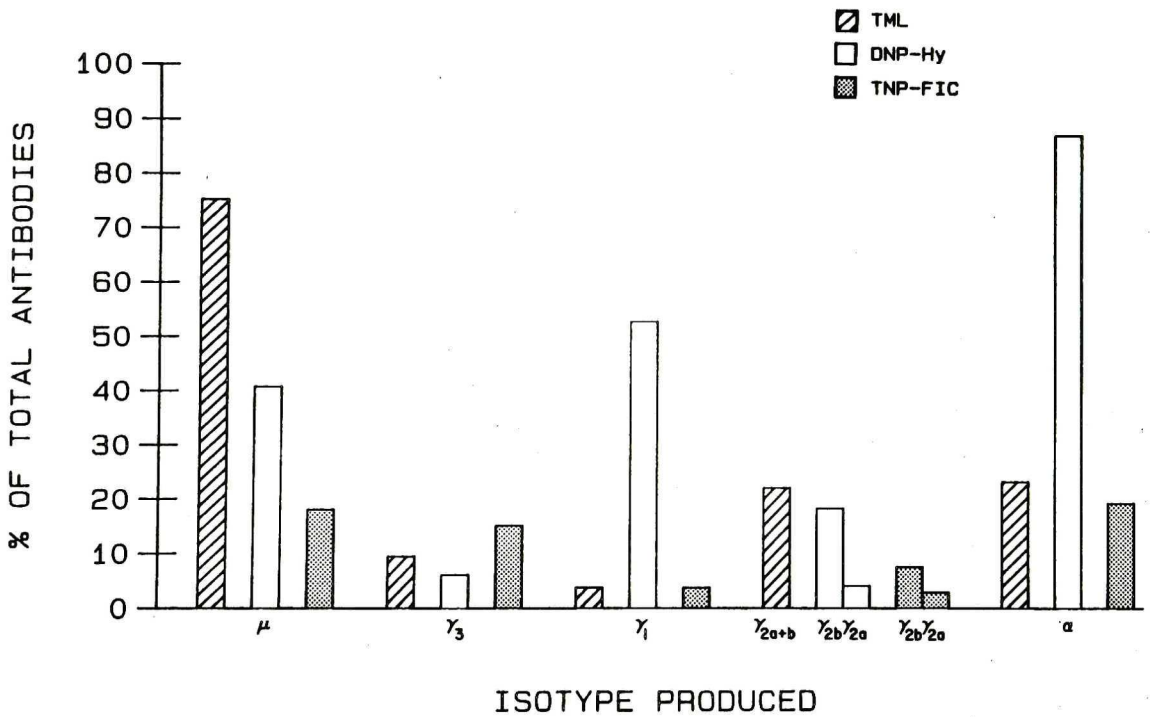

Figure 8. Comparison of the relative frequencies of heavy chain isotypes produced by primary TML-specific B-cell clones with those produced by primary B cells responsive to a TD (DNP-Hy) and a TI-2 (TNP-FIC) antigen. Anti-TML antibodies were analyzed with class-specific reagents as described in Materials and Methods. The relative frequency of each isotype produced in all three responses is expressed as the percent of total clones which secrete that particular isotype. Anti-DNP and anti-TNP antibody data are from Teale et al. (1981) and Mongini et al. (1983), respectively.

account for both the lack of IgG1 produced and the small precursor frequency observed in the overall response to TML. However, the isotype profile of the TML-specific response is very similar to the profile observed in the HA-specific response, as shown in Figure 9. Studies from other investigators have shown that the stimulation of primary HA-specific B cells in the splenic focus system, as well as in another experimental system, is exquisitely dependent upon the presence of antigen-primed recipient T cells (Cancro et al., 1978, Pierce et al., 1978, Virelizier et al., 1974). Interestingly, the isotype profile of the HA-specific response is somewhat different from that of the DNP-Hy stimulated response, particularly in the occurrence of the IgG1 isotype. This isotype is infrequently produced in the HA-specific response. Furthermore, the predominant isotype in the HA-specific response is also IgM. Although IgA occurs more frequently in the HA-specific response than in the TML-specific response, the isotype distribution of monoclonal antibodies in the responses to these two different natural antigens are remarkably alike. This suggests that infectious agents may stimulate B cells by pathways which are different from those for chemically defined antigens. However, it is possible that these infectious agents may stimulate B cells by the same pathways. Because TML and HA are complex antigens composed of a wide array of antigenic determinants, it is possible

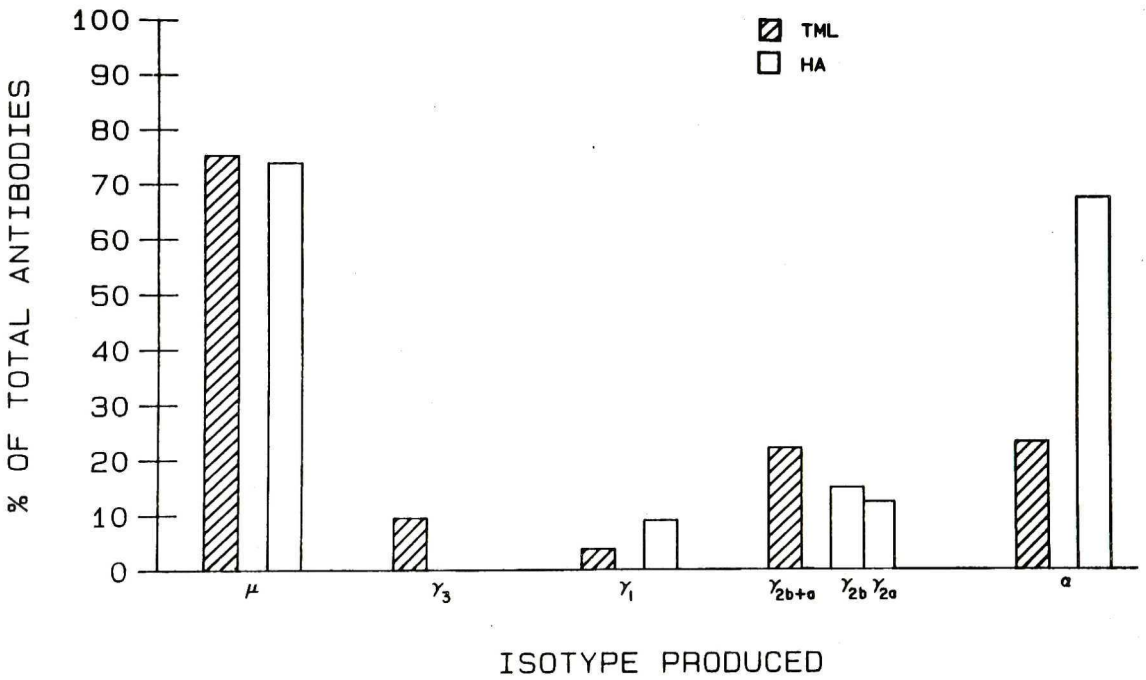

Figure 9. Relative frequencies of heavy chain isotypes produced by primary TML-specific and HA-specific B-cell clones. Anti-TML antibodies were analyzed with class-specific reagents as described in Materials and Methods. The relative frequency of each isotype produced in both responses is expressed as the percent of total clones which secrete that particular isotype. Anti-HA antibody data is from Wylie and Klinman (1981).

that many of the determinants contained within the intact bacterium and virus are presented to the host in suboptimal concentrations or form compared to chemically defined antigens. Therefore, B cells specific for these determinants may not be optimally stimulated. Consequently, particular isotypes may not be optimally produced.

Table 13 shows the number of different isotypes secreted by individual clones from primary TML-specific clones. The majority of these clones secrete only 1 isotype, but a few clones secrete 2 to 4 isotypes. It is important to note that all experiments herein were designed to ensure that each splenic fragment culture contained either 0 or 1 TML-specific B cell (Figure 3). The Poisson distribution predicts that less than 1% of the responding spleen fragments may contain more than a single TML-specific B cell. Since Table 13 indicates that almost 30% of the fragments produce antibodies of 2 or more isotypes, it is likely that the majority of fragments which secrete more than 1 heavy chain class contain only one TML-specific B cell. In contrast to the TML-specific response, most clones from primary B cells stimulated by DNP-Hy or TNP-FIC secrete more than 1 isotype with a significant proportion of clones secreting 3 to 5 (6, in the case of TNP-FIC) different isotypes (Mongini et al., 1982, 1983; Teale et al., 1981).

The data presented in Table 14 show a more detailed analysis of the complexity of isotype expression in the

TABLE 13

NUMBER OF ISOTYPES SECRETED BY CLONES

FROM PRIMARY TML-SPECIFIC CELLS

No. Clones Analyzed[a]	% of Individual Clones Secreting the Following No. of Different Isotypes:				
	1	2	3	4	5
158	72.2	21.5	5.7	0.6	0.0

a) TML-specific clones from unprimed CBA/Ca donor spleen cells were obtained from fragment cultures derived from AKD-TML primed AKR or B10.BR recipients.

TABLE 14

ANALYSIS OF THE NUMBER OF ISOTYPES PRODUCED IN FRAGMENTS SECRETING GIVEN ISOTYPES OF ANTI-TML ANTIBODY[a]

Fragments Secreting the Following Isotype	No. of Clones	% of Individual Clones Secreting the Following No. of Isotypes: 1	2	3	4	5	$\bar{X}$ No. Isotypes per Fragment
IgM	119	71.4	21.8	5.9	0.8	0.0	1.36
IgG3	15	26.7	60.0	6.7	6.7	0.0	1.94
IgG1	6	0.0	33.3	50.0	16.7	0.0	2.83
IgG2	35	34.3	48.6	17.1	0.0	0.0	1.83
IgA	37	35.1	43.2	18.9	2.7	0.0	1.79

a) TML-specific clones from unprimed CBA/Ca spleen cells were obtained from fragment cultures derived from AKD-TML primed AKR or B10.BR recipients. Total number of clones analyzed = 158.

anti-TML antibodies secreted by primary TML-specific clones. The percent of fragments which secreted anti-TML antibody of a particular isotype or of 1 to 4 additional isotypes is indicated. The data show that clones which secrete any one of the isotypes, except IgM, secrete more than 1 other isotype. For example, of the 119 clones which secrete IgM, 71.4% secrete IgM alone while 21.8% of these IgM secretors also secrete one other isotype. In contrast, clones which secrete IgG1 tend to secrete most of the other isotypes. None of the 158 clones secreted IgG1 only. These results are graphically illustrated in Figure 10 where the percent of those fragments secreting anti-TML antibody of a particular isotype (Column A) which also secrete anti-TML antibody of each additional isotype (Column B) is shown. Collectively, the heavy chain class analysis data indicate that the majority of the clones from primary TML-specific B cells secrete IgM with a substantial proportion of these secreting solely IgM. However, when clones secrete other isotypes, these clones frequently secrete more than 1 isotype. Previous studies have indicated that clones which secrete IgG, IgE, or IgA isotypes after stimulation by either TD antigens or mitogens arise from B cells which express solely IgM on their surface (Teale et al., 1981; Andersson et al., 1978). The results reported in this thesis are consistent with this view since a significant number of the TML-specific

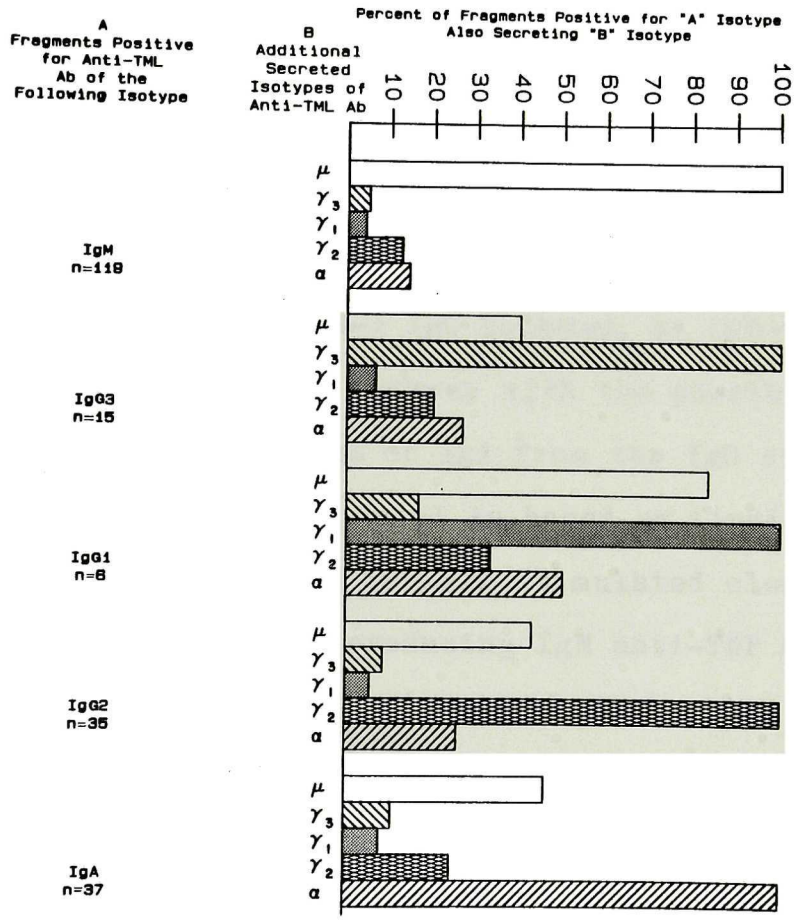

Figure 10. Analysis of anti-TML (Ab) isotype co-expression by primary CBA/Ca B-cell clones. TML-specific clones from unprimed CBA/Ca spleen cells were obtained from fragment cultures derived from AKD-TML primed AKR or B10.BR recipients. The isotype(s) of the antibody produced by B-cell clones was determined by RIA using whole TML organisms as the immunoadsorbent and rabbit anti-mouse heavy chain class-specific sera. The total number of clones analyzed = 158.

clones which secrete IgG subclasses or IgA also secrete IgM. Thus, it is likely that most clones which secrete multiple immunoglobulin heavy chain classes of anti-TML antibody arose from a single IgM-positive precursor. This also appears to be the case in responses to TNP-FIC (Mongini et al., 1982, 1983). Moreover, from the findings of this latter study, Mongini et al. (1983) proposed three distinct switching pathways employed by B cells responding to TNP-FIC: a common IgM-> IgG pathway, an IgM->IgE pathway, and an IgM->IgA pathway with the possibility of occasional switches to IgE or IgA from the IgG switching pathway. This switching model is based on findings in the splenic focus system that TNP-FIC stimulated clones have a high probability of also producing IgM anti-TNP antibody and anti-TNP antibody of each IgG subclass whose IgCH gene is encoded 5', but not 3', to the gene for the given subclass. However, only a small proportion of the fragments that secreted IgE or IgA anti-TNP antibody also secreted anti-TNP antibody of any given 5'-encoded IgG isotype. [The 5' to 3' IgCH (CH) gene sequence located on the 12th chromosome in mice is: 5'-Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, Cα-3'; Shimizu et al., 1981, 1982; Liu et al., 1980a]. In contrast to the TNP-FIC response, primary anti-TML antibody of the IgG subclasses does not have a high propensity for co-expression of isotypes encoded by 5' Cγ genes. Since there are low levels of co-expression of 5'-encoded IgG subclasses, the common IgM->IgG switching

pathway probably occurs to some extent in the TML-specific antibody response. However, from the data presented, it appears that other switching pathways account for the majority of the anti-TML antibody response. The data suggest distinct switching pathways for each IgG subclass. It is possible, however, that because these antibodies were harvested much later in the response than those in the TNP-FIC stimulated response (16-22 days and 10-17 days after antigen stimulation, respectively) the 5'-encoded IgG isotypes were expressed early in the response, before antibody harvest.

3. Isotypes secreted by memory B cells.

It was of interest to determine the isotype profile of memory TML-specific B cells to establish whether particular heavy chain classes are selectively increased after antigen exposure. Such a finding would suggest an important role for such isotypes in the memory response to S. typhimurium infections. Furthermore, the isotype profile may distinguish the memory TML-specific B-cell pool from the primary B-cell pool since prior antigen exposure should cause both proliferation and differentiation of TML-specific B cells. Hence, spleen cells from CBA/Ca mice previously primed with AKD-TML were analyzed in the splenic focus system. Table 15 shows that, of the 208 memory anti-TML-responsive clones analyzed, the predominant isotype produced was IgM, which paralleled the isotype profile of

TABLE 15

PROPORTION OF MEMORY TML-SPECIFIC B-CELL CLONES FROM CBA/Ca MICE WHICH SECRETE A PARTICULAR ISOTYPE

No. Clones Analyzed[a]	% of clones secreting:[b]				
	IgM	IgG3	IgG1	IgG2	IgA
208	71.6	23.1	35.6	57.7	44.7

a) Memory clones represented a pool of secondary and tertiary TML-specific clones which were obtained from AKD-TML primed CBA/Ca spleen cells in the splenic focus system as described in Table 26.

b) The isotype of the antibody produced by memory clones was determined by RIA using whole TML organisms as the immunoadsorbent and rabbit anti-mouse heavy chain-specific sera. There was no difference in the isotype profile of secondary and tertiary clones.

the primary anti-TML response. In contrast, all other isotypes increased from 2- to 10-fold over the primary response (compare the results in Table 12 and Table 15). Although memory anti-TML B cells of the IgG1 isotype are not as predominant as they are in the DNP-Hy memory response (35.6% as opposed to 95.6%; Table 15 and Teale et al., 1981), the IgG1 response does increase significantly after immunization with TML (from 3.9% to 35.6%). However, IgG1 from DNP-specific memory B cells increases less than 2-fold over the primary DNP-specific response. Figure 11 compares the relative frequencies of isotypes produced by memory TML-specific clones to those produced by DNP-Hy stimulated memory B cells. Whereas the differences in isotype profiles were more apparent between the primary TML-specific response and the primary DNP-Hy stimulated response (Figure 8), the memory B-cell response profiles are more similar.

Though the predominant isotype in the memory TML-specific response is IgM, Table 16 shows that the majority of the IgM anti-TML antibody is secreted in conjunction with most of the other isotypes. Only a small fraction of the total memory clones secrete only IgM. This small subset of clones secreting only IgM could possibly be attributed to the stimulation of primary B cells in the memory B-cell pool. If this were true, it is possible that upon further stimulation these cells would differentiate

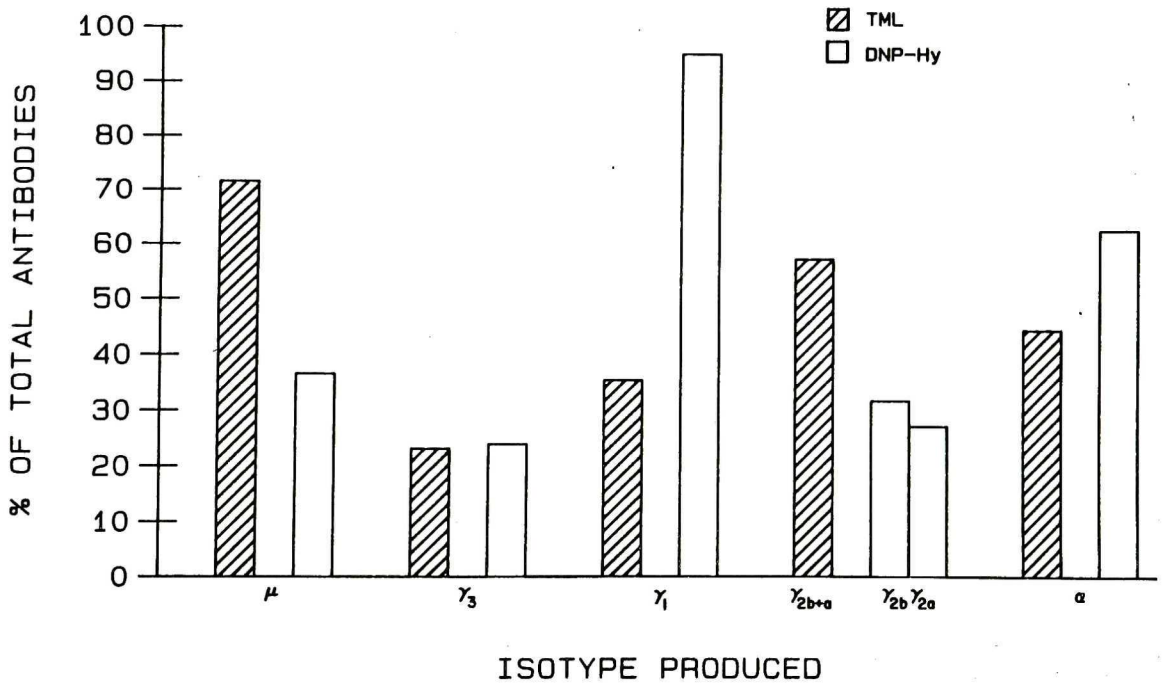

Figure 11. Relative frequencies of heavy chain isotypes produced by TML-specific and DNP-specific memory B-cell clones. Anti-TML antibodies were analyzed with class-specific reagents as described in Materials and Methods. The relative frequency of each isotype produced in both responses is expressed as the percent of total clones which secrete that particular isotype.

TABLE 16

ANALYSIS OF THE NUMBER OF ISOTYPES PRODUCED IN FRAGMENTS SECRETING GIVEN ISOTYPES OF ANTI-TML ANTIBODY[a]

Fragments Secreting the Following Isotype	No. of Clones	% of Individual Clones Secreting the Following No. of Isotypes: 1	2	3	4	5	$\bar{X}$ No. Isotypes per Fragment
IgM	149	16.8	36.9	26.2	14.1	6.0	2.56
IgG3	48	2.1	20.8	29.7	29.2	18.8	3.44
IgG1	74	4.1	29.7	25.7	28.4	12.2	3.15
IgG2	120	13.3	30.8	28.3	20.0	7.5	2.77
IgA	93	12.9	19.4	32.3	25.8	9.7	3.00

a) The pool of secondary and tertiary TML-specific clones analyzed were derived from AKD-TML primed CBA/Ca mice as described in Table 26. Total number of clones analyzed = 208.

into memory B cells capable of secreting IgG1. Thus, the proportion of IgG1 to IgM in the memory TML-specific B-cell pool would approach that of the memory DNP-Hy responsive B-cell pool (Figure 11). However, it is likely that this type of stimulation pattern may prove to be a typical pattern in responses to infectious disease agents and different than the pattern typically observed after analysis of chemically defined antigens, such as DNP. Table 16 also indicates that all isotypes, on the average, are expressed with 2 other isotypes. Further, a significant proportion of clones secreting each isotype secrete 3 and 4 additional isotypes. In contrast, the majority of primary TML-specific B-cell clones, on the average, secrete only 1 additional isotype (compare Table 14 and Table 16). These findings indicate that primary TML-specific B cells do not undergo as many heavy chain class switches as memory B cells, and these data are consistent with the findings in the DNP-Hy system (Teale et al., 1981).

4. Fine specificity analysis of TML-specific B cells in spleens of CBA/Ca mice

The outer membranes of gram-negative bacteria contain a variety of complex antigenic determinants, each of which is potentially able to elicit a specific antibody response. Since the anti-TML antibodies were detected in an RIA using whole TML organisms as the immunoadsorbent,

the antibody specificities of the TML-specific clones were unknown. However, the LPS molecule is considered to be one of the major antigenic determinants on salmonella organisms. A major component of the anti-S. typhimurium antibodies in sera of mice immunized with live TML organisms is directed against LPS (Metcalf and O'Brien, 1981). In addition, the O-antigen portion of the salmonella LPS molecule is a major virulence factor for this bacterium (Germanier, 1970, 1972). Thus, to determine whether the majority of the clones produced after stimulation with AKD-TML were specific for LPS, TML-LPS and S. pneumoniae were used as immunoadsorbents in the RIA. As indicated in Table 17, only 24.5% of 171 TML-specific antibodies analyzed were directed against the LPS molecule. Although S. pneumoniae was included as a negative specificity control, an equal proportion of clones (22.2%) was also reactive to this gram-positive bacterium. These clones may recognize a common determinant between the two bacteria. Interestingly, the proportion of TML-specific clones reactive to S. pneumoniae does not increase upon secondary immunization. Further, the frequency of S. pneumoniae-specific clones relative to that of LPS-specific clones decreases (E.S. Metcalf, unpublished observation). More important, as shown in Table 18, 96.6% of the memory anti-TML antibody-producing clones are reactive with LPS, as opposed to only 28.6% of all the TML-specific primary clones examined. In addition, the frequency of memory TML-

TABLE 17

REACTIVITY OF PRIMARY TML-SPECIFIC CLONES

Reactivity Pattern No.	Reactivity in RIA with:[a] TML	LPS	S. pneumoniae	No. of Clones[b]
1	+	+	-	30
2	+	-	-	103
3	+	+	+	12
4	+	-	+	26

a) Anti-TML positive clones in fragment cultures derived from primed AKR or B10.BR recipients were assayed by RIA with either TML-LPS or Streptococcus pneumoniae as the immunoadsorbent.

b) Total number of clones analyzed = 171.

TABLE 18

COMPARISON OF THE LPS-REACTIVITY OF

PRIMARY AND MEMORY TML-SPECIFIC CLONES

Donor Cells	Total No. of Clones Analyzed	% of Anti-TML Antibody Clones Reactive with TML-LPS[a]
Unprimed	420	28.6
TML Primed	208	96.6

a) Primary and memory anti-TML positive clones in fragment cultures derived from AKD-TML primed AKR or B10.BR recipients were assayed by RIA using TML-LPS as the immunoadsorbent.

specific cells is between 123-178 per 10^6 splenic B cells (see below). These findings indicate that the precursor frequency expands 12- to 18-fold (see Tables 11 and 26) after antigen exposure with an apparent selective expansion of LPS-specific clones. These results corroborate the findings in the influenza system in which the HA is one of the major antigenic determinants expressed by this virus. After immunization, a 10- to 20-fold increase in the PR8-specific B-cell repertoire occurs and there appears to be a selective expansion of HA-specific B cells from 30% to 80%. (Cancro et al., 1978; Gerhard et al., 1975). In contrast, only a 2- to 4-fold increase in precursor frequency occurs after immunization with simple haptenic determinants such as DNP (Klinman, 1972).

The LPS molecule is composed of three distinct regions (Figure 12): the O-antigen region, the basal core region, and the lipid A region (Lüderitz et al., 1971). The O-antigen region consists of repeating units of oligosaccharides which contain specific sugars in specific linkages. Consequently, this region of the molecule permits the classification of different Salmonella species into various serogroups. The O-antigen structure for S. typhimurium consists of repeating units of a branched tetrasaccharide of abequose, mannose, rhamnose, and galactose and, thus, belongs to the Kauffman-White serological group B (Lüderitz et al., 1971). Furthermore,

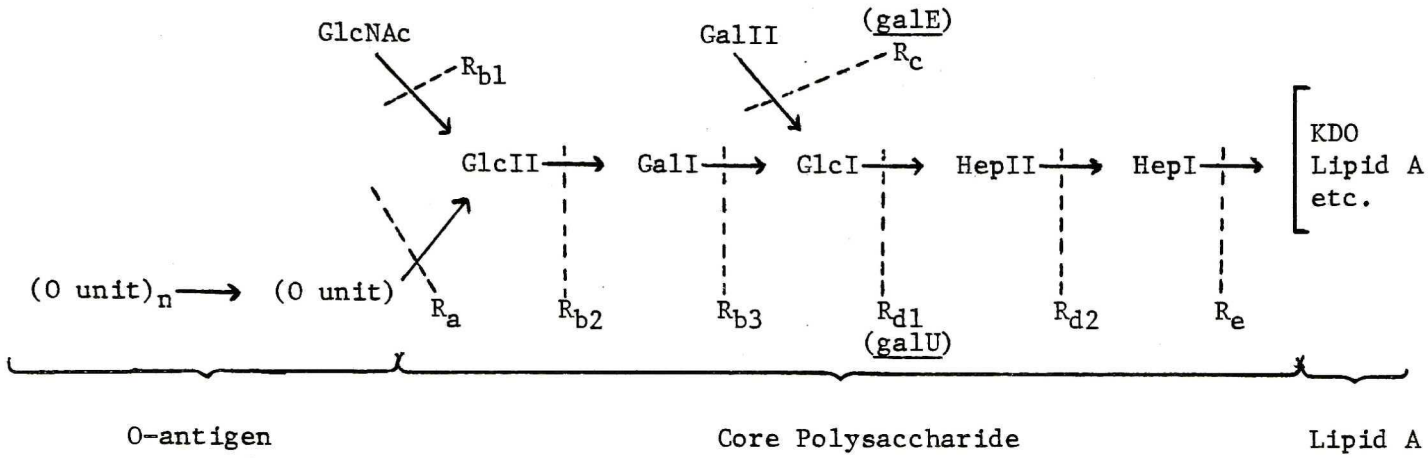

Figure 12. Salmonella typhimurium lipopolysaccharide structure. GlcNAc, N-acetyl-D-glucosamine; Glc, D-glucose; Hep, L-glycero-D-mannoheptose; KDO, 3-deoxy-D-mannooctulosonic acid (2-keto-3-deoxyoctonic acid). Dashed lines represent points where LPS synthesis is blocked in various rough mutants. (Modified from Hudson et al., 1978).

TML expresses the O-antigenic determinants 1, 4, and 12 (Elkins and Metcalf, 1984). Organisms which contain the entire LPS molecule are referred to as smooth strains. Rough mutant strains are blocked at different points in the biosynthesis of the LPS molecule. These mutants have been classified into chemotypes, Ra through Re, based on the sugar composition of their polysaccharides. Figure 12 shows that the Ra polysaccharide represents the complete basal core of the parental, smooth, polysaccharide, whereas the Rb to Re mutants produce defective cores. The Re mutant contains the most defective polysaccharide, composed only of 2-Keto-3-deoxyoctonic (KDO) acid and lipid A. Michael and Mallah (1981) have shown that a smooth strain of S. minnesota can absorb out antibodies in murine anti-rough mutant serum, which suggested that smooth strains have smooth (O-antigen) as well as rough antigenic determinants on their surface. Therefore, TML-specific clones found to be reactive against LPS were assayed against a series of S. typhimurium rough mutants in the RIA. This analysis should indicate which subregions of the LPS molecule elicit antibody as well as permit the determination of a pattern of reactivity of these LPS-specific clones. The results presented in Table 19 show that 72.1% of the primary anti-TML-LPS antibodies do not react with any of the rough mutants tested. This high percentage of non-binding can not be accounted for by insufficient coating of wells by the immunoadsorbent. An

TABLE 19

FINE SPECIFICITY OF PRIMARY AND SECONDARY LPS-REACTIVE CLONES FROM CBA/Ca MICE[a]

Donor Cells	No. Clones Analyzed	Immunoadsorbent	% Total LPS+ Clones with the Following Reactivity Pattern					
		LPS	+	+	+	+	+	+
		Ra	-	+	+	+	+	+
		Rc	-	-	+	+	+	+
		Rd1	-	-	-	+	+	+
		Rd2	-	-	-	-	+	+
		Re	-	-	-	-	-	+
Primary	104		72.1	0.0	0.0	1.9	0.0	26.0
Memory	199		43.7	2.0	1.5	3.0	5.5	44.2

a) Primary and memory anti-TML positive clones reactive with LPS were assayed by RIA using whole S. typhimurium rough mutant organisms as immunoadsorbents.

anti-flagellin antibody, which binds efficiently to each rough mutant immunoadsorbent, was included as a positive control. Most of the other primary LPS-specific antibodies bound to all rough mutants. Out of 104 clones tested, only 2 were directed to a region within the inner core, somewhere on the heptose II molecule. These results suggest that the majority of the primary LPS-specific B cells are directed against the O-antigen portion of the molecule since they bound only to LPS and not to the O-antigen-deficient mutants. Further, very few B cells are directed against sites within the core polysaccharide region of the LPS molecule. The remainder of the LPS-specific B cells are directed against sites on the KDO region of the inner core or possibly the lipid A region itself. Insufficient quantities of anti-LPS antibody from primary TML-specific clones precluded testing their reactivity against lipid A. However, recent studies from our laboratory (M. Gaffney and E.S. Metcalf, unpublished observations) have demonstrated that the majority of secondary TML-specific B-cell clones which react with all the rough mutants listed in Table 19 do react with lipid A. Therefore, the results presented in Table 19 suggest that one quarter of the primary TML-specific response may be specific for lipid A.

For comparison, Table 19 also shows the reactivity pattern of anti-LPS antibodies from memory TML-specific

clones. These results indicate that, in contrast to the primary response, less than half of the memory anti-LPS antibody response is directed against the O-antigen region. A small proportion of the antibodies is directed against various sites within the core polysaccharide region. Moreover, almost half of the antibodies are directed to the KDO or lipid A region. Therefore, after antigen exposure, the TML-specific B-cell repertoire contains equal numbers of the O-antigen-specific and the putative lipid A-specific B-cell subsets.

The outer membrane of S. typhimurium, in addition to LPS, contains proteins and phospholipids (see Figure 13). It was shown in Table 18 that only 28.6% of the primary TML-specific B cells are directed against the LPS molecule. Hence, the remaining 71.4% of the response are most likely directed against these other outer membrane components. In an effort to determine the specificity of the LPS-negative clones, culture fluids from these clones were re-assayed using different, purified S. typhimurium surface components as immunoadsorbents. These components included: polymerized flagellin (POL)[1]; 34K porin, a major outer membrane protein of salmonellae; and lipid A. Table

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[1] Flagella of Salmonella, which are associated with both the outer and inner cytoplasmic membranes, can be dissociated by acid into its component protein monomers, flagellin. The subunits can be re-polymerized in vitro to form POL which is indistinguishable from native flagella (Ada et al., 1964).

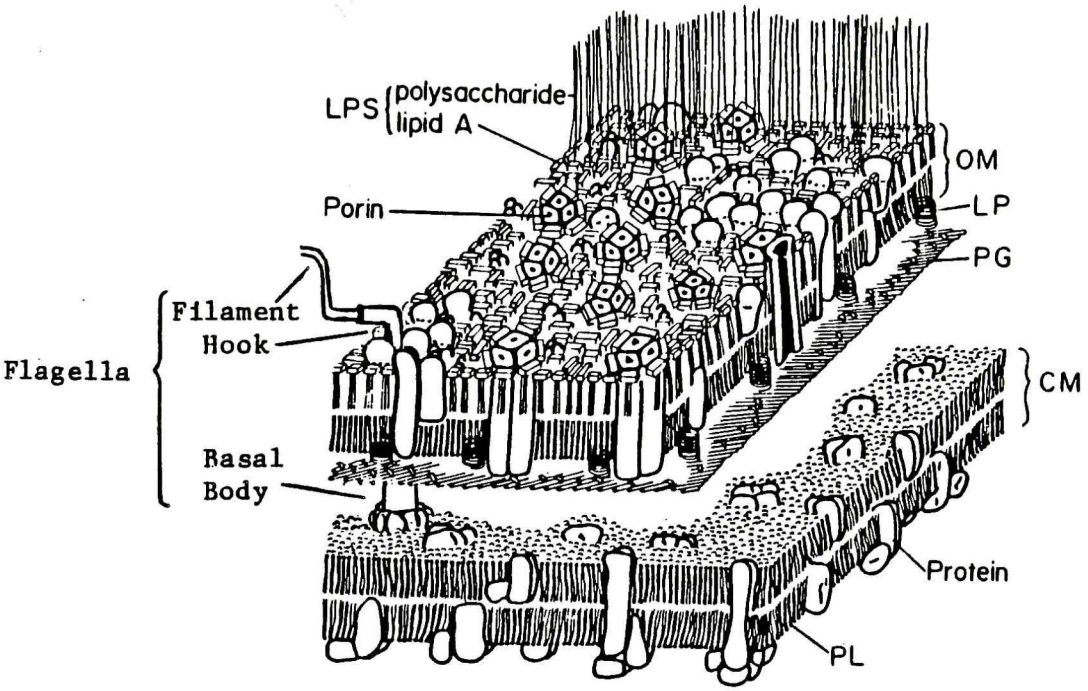

Figure 13. Diagram of the gram-negative bacterial envelope structure. LPS, lipopolysaccharide; OM, outer membrane; LP, lipoprotein; PG, peptidoglycan; CM, cytoplasmic membrane; PL, phospholipid. (Modified from DiRienzo et al., 1978).

20 shows that of 187 primary TML-specific clones that did not react with LPS, $\leq$ 3% to 8% reacted with one of the other surface determinants tested. The majority of these antibodies do not react with any of these determinants. Again, lack of reactivity is not due to failure of the immunoadsorbent to effectively coat wells of the microtiter plates. Positive antibody controls for each immunoadsorbent demonstrated that significant antigen binding occurred. Of some interest is the observation that almost 25% of the clones can bind to more than one surface antigen. In fact, 29 of the clones react with all three antigens. These results suggest that flagella, porins, and/or lipid A can twist or fold about each other and that a small proportion of primary TML-specific B cells recognize a determinant produced by the combination of the flagella and porin, the flagella and lipid A, the porin and lipid A, or all three antigens. Furthermore, these anti-TML-antibodies have the capacity to partially bind to each of the purified antigens which compose the combinational determinants. It is surprising to find that so few TML-specific clones react with POL. Flagella are long, fine filamentous appendages which extend out from the outer membrane surface. Thus, they should be readily accessible to antibody precursor cells for stimulation. Moreover, anti-flagellin antigen (anti-H) antibody titers have been found to significantly increase after immunization with AKD

TABLE 20

FINE SPECIFICITY OF NON-LPS-REACTIVE

CLONES FROM CBA/Ca MICE

Donor Cells	No. Clones Analyzed	Immuno-adsorbent	% Total LPS-Clones with the following Reactivity Pattern[a]							
		POL	+	-	-	+	+	-	+	-
		Porin	-	+	-	+	-	+	+	-
		Lipid A	-	-	+	-	+	+	+	-
Primary	187		7.5	8.0	2.7	5.3	2.7	1.1	15.5	57.2
Memory	5		40	0	0	40	0	0	0	20

a) TML-specific clones which did not react with LPS were re-assayed by RIA using POL, porin protein, or lipid A as the immunoadsorbent according to the procedure described in Materials and Methods.

Salmonella vaccines in field trials (Levine and Hornick, 1981). Another unexpected result is the observation that few TML-specific clones react with porin since these proteins are also exposed above the membrane surface. However, it is less surprising to find very few clones reactive with lipid A. Lipid A is the innermost moiety of the LPS molecule. Thus, the O-antigen region and/or the polysaccharide region of the molecule may mask the expression of lipid A antigenic determinants. The bacterium may, however, be processed by the host in such a way as to expose lipid A antigenic sites. These sites would not necessarily cross-react with determinants of the intact, free LPS molecule. This would account for the few lipid-A specific clones indicated in Table 20.

Because LPS negative clones from memory TML-specific B cells represent only 3.4% of the response, very few clones were available for further analysis. Thus, the data from these clones shown in Table 20 must remain inconclusive. However, if these 5 clones are representative of the remainder of the memory response, then it may be that flagella-specific B cells are selectively expanded after antigen exposure rather than those B-cell subsets responsive to determinants other than LPS.

5. Analysis of Peyer's patch primary TML-specific B cells

The natural site of S. typhimurium infection is the gastrointestinal tract. Peyer's patches compose a significant portion of the gut-associated lymphoid tissue (Parrot, 1976) and contain antigen-sensitive T and B cells and accessory cells (Kagnoff and Campbell, 1974; Kiyono et al., 1982). Thus, it was predicted that the Peyer's patches may have a higher TML-specific precursor frequency than the spleen. However, as preliminary results from one experiment show in Table 21, there is no significant difference ($p>0.15$) in the frequency of TML-specific spleen cells and Peyer's patches cells. These results are consistent with the findings of Gearhart and Cebra (1979). These investigators previously observed comparable frequencies in primary B cells responsive to PC-Hy found in Peyer's patches and in the spleen. These investigators did, however, find differences in the fine specificity and isotype distribution of PC-specific B-cell clones between these two lymphoid tissues. Since extensive analysis of clones by RIA required antibody of sufficient concentration to allow testing in several different assays, only three quarters of the clones from splenic B cells and half of the clones from Peyer's patches could be assessed for isotype and fine specificity. Because of this, the isotype analysis of primary TML-specific clones from the spleen, presented in Table 22, is somewhat limited and slightly

TABLE 21

FREQUENCY OF PRIMARY TML-SPECIFIC B CELLS

IN THE SPLEEN AND PEYER'S PATCHES OF CBA/Ca MICE

Donor	Cell Source	Total Number of Cells Transferred (X 10^6)	No. TML-Specific Clones per 10^6 Cells Transferred[a]	Number of Clones Analyzed
CBA/Ca	Spleen	80	0.19	15
CBA/Ca	Peyer's Patches	55	0.24	13

a) 40 x 10^6 donor spleen cells or 5-20 x 10^6 donor Peyer's patches cells were injected into AKD-TML primed B10.BR recipients. Priming and stimulation of recipients and detection of clones by RIA were as described in Figure 7. As assessed by Student's t-test, the number of clones in the Peyer's patches was not significantly ($p > 0.15$) different from the number in the spleen.

TABLE 22

PROPORTION OF CLONES FROM PRIMARY TML-SPECIFIC SPLENIC AND PEYER'S PATCHES B CELLS SECRETING PARTICULAR ISOTYPES[a]

Cell Source	No. Clones Analyzed	% of Clones Secreting: IgM	IgG3	IgG1	IgG2	IgA
Spleen	11	63.6	0	0	27.3	45.5
Peyer's Patches	6	0	0	0	83.3	16.7

a) The isotype of the antibody produced by primary splenic and Peyer's patches clones was determined by RIA using whole TML organisms as the immunoadsorbent and rabbit anti-mouse heavy chain-specific sera.

skewed from the pattern presented in Table 12. Nonetheless, the pattern is generally representative of the splenic isotype profile, as well as, distinctly different from that observed in the Peyer's patches. Of interest is the finding that a lower proportion of clones from Peyer's patches B cells produce IgA relative to clones from splenic B cells. Previous studies have shown that Peyer's patches generate a high proportion of plasma cells expressing IgA compared with B cells from other tissues (Craig and Cebra, 1971; Jones et al., 1974; Cebra et al., 1977). Gearhart and Cebra (1979) demonstrated in the splenic focus system that the predominant isotype secreted by both PC-specific and inulin-specific B cells from Peyer's patches was IgA. Further, both these antigens are found on intestinal bacteria. Thus, the findings in Table 22 are unexpected. However, only 6 clones were available for analysis. With so few clones, it is difficult to draw firm conclusions about the isotype distribution of anti-TML antibodies from Peyer's patches B cells. It is possible that upon examination of additional clones the proportion of IgA-producing clones will increase. On the other hand, Fuhrman and Cebra (1981) reported that anti-cholera toxoid precursor cells do not show high proportions of cells in the Peyer's patches secreting IgA. As in the spleen, the isotype profile of Peyer's patches TML-specific clones may prove to be different than that of B cells of other

specificities.

The fine specificity of primary TML-specific clones derived from spleen and Peyer's patches is shown in Table 23. As opposed to 36.4% of the splenic TML-specific clones, none of the anti-TML antibodies from Peyer's patches B cells are directed against the LPS molecule. Nevertheless, the reactivity pattern of the Peyer's patches-derived clones is comparable to that of LPS negative splenic clones. The majority of the clones do not react with any of the antigenic determinants tested. Again, there are too few clones to draw definite conclusions. It remains to be seen how similar or different TML-specific subsets are within the spleen and Peyer's patches. It is possible that distinctions between the TML-specific B-cell repertoires in these two tissues may be identified only after these clones have been analyzed against a wider variety of defined determinants.

C. Analysis of the TML-Specific B-Cell Response in CBA/N Mice

1. Serum antibody responses to AKD-TML

CBA/N mice have an X-linked immune defect which is associated with the absence of a mature subpopulation of B cells (Huber *et al.*, 1977; Ahmed *et al.*, 1977; Subbarao *et al.*, 1979; Metcalf *et al.*, 1980). The *xid* gene which confers this B-cell defect also confers the susceptibility of CBA/N mice to *S. typhimurium* (O'Brien *et al.*, 1979). In

TABLE 23

COMPARISON OF THE FINE SPECIFICITY OF PRIMARY TML-SPECIFIC CLONES IN THE SPLEEN AND IN THE PEYER'S PATCHES OF CBA/Ca MICE

Cell Source	No. Clones Analyzed	% LPS Negative[a]	Immuno-adsorbent	% LPS⁻ Clones with the following Reactivity Pattern[b]							
			POL	+	−	−	+	+	−	+	−
			Porin	−	+	−	+	−	+	+	−
			Lipid A	−	−	+	−	+	+	+	−
Spleen	11	63.6		0	42.9	0	0	0	0	14.3	42.9
Peyer's Patches	6	100		0	33.3	0	0	0	0	0	66.7

a) Percent of total anti-TML antibody response which are not reactive with TML-LPS.

b) TML-specific clones which did not react with LPS were re-assayed by RIA using POL, porin protein, or lipid A as the immunoadsorbent according to the procedure described in Materials and Methods.

addition, immune-defective CBA/N mice have low pre-immune serum IgM and IgG3 levels (Amsbaugh et al., 1972; Perlmutter et al., 1979). Previous studies by O'Brien et al. (1981) demonstrated that both salmonella-resistant, immunologically normal (CBA/N x DBA/2N)F_1 female and salmonella-susceptible immune-defective F_1 male mice make IgM anti-TML serum antibodies after immunization with AKD-TML; however, IgG titers at 3 and 4 weeks after immunization were much lower in the F_1 male mice. To further characterize the anti-TML antibody response in immune-defective mice, serum samples from CBA/N mice and CBA/Ca controls were analyzed by RIA to determine the LPS specificity and the isotypes 4 weeks after immunization with 5 x 10^8 AKD-TML organisms. Panel A of Figure 14 shows the geometric mean titers of anti-TML kappa chain, IgG (all subclasses), and IgM antibodies in CBA/Ca and CBA/N mice. These results suggest that the anti-TML IgG response in CBA/N mice is significantly ($p<0.05$) reduced when compared with CBA/Ca mice; the data are also consistent with the results of O'Brien et al. (1981) and Metcalf et al.[2] In addition, the majority of the anti-LPS antibody activity (Figure 14, Panel B) is in the IgG fraction in CBA/Ca mice

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[2] Metcalf, E.S., M. Gaffney, D. Weinstein, K. Elkins, and A.D. O'Brien. Mechanisms of immunity to Salmonella typhimurium: analysis of vaccinated salmonella-susceptible xid mice. Manuscript in preparation.

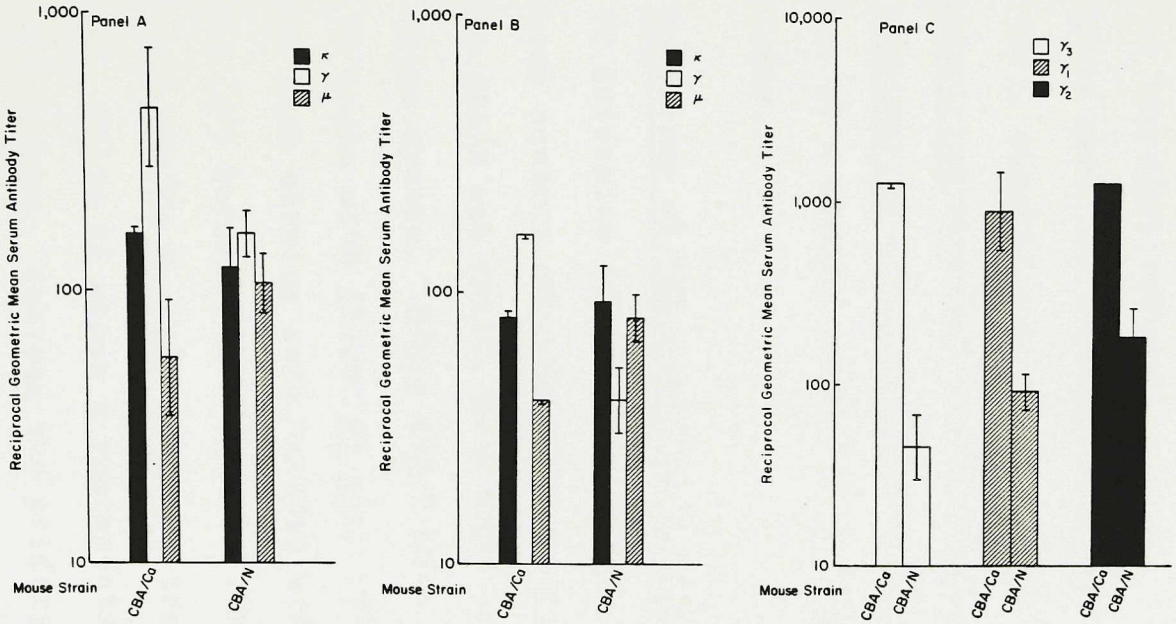

Figure 14. Five CBA/N and 2 CBA/Ca mice were immunized i.v. with 5 x 10^8 AKD-TML organisms. Four weeks after immunization, serum from each mouse was assayed by RIA using whole TML organisms (Panel A) or TML-LPS (Panels B and C) as the immunoadsorbents. Anti-TML antibodies were detected with rabbit anti-mouse kappa chain, IgG (all subclasses), and IgM sera (Panels A and B) and with rabbit anti-mouse IgG3, IgG1, and IgG2 sera (Panel C). Geometric mean titers $\pm$ 2 standard errors are given.

and in the IgM fraction in CBA/N mice. Moreover, subclass analysis of the IgG serum component of the anti-LPS antibody response (Figure 14, Panel C) shows that all subclasses are reduced ($p<0.05$) in CBA/N mice, but the IgG3 fraction is the most reduced.

These results, together with the results of O'Brien et al. (1981), suggest that the susceptibility of xid mice to S. typhimurium is a result of a delayed and diminished anti-S. typhimurium antibody response which could be a consequence of a reduced number of S. typhimurium-specific B-cell clones.

2. Frequency of TML-specific B cells

To determine if, in fact, immune-defective mice have reduced numbers of salmonella-specific B cells, spleen cells from CBA/N and CBA/Ca mice were compared in the splenic focus system. Since CBA/N mice have fewer splenic B cells to begin with (Scher et al., 1975), donor spleen cells from both strains were treated with anti-Thy 1 and complement (C') before cell transfer. Thus, equal numbers of CBA/N and CBA/Ca B cells could be transferred to recipients. Table 24 shows a representative example of such an experiment. Whereas the primary TML-specific precursor frequency in CBA/CA spleens is 6.68 per 10^6 B cells, in this experiment, not a single TML-specific clone was detected in 225 x 10^6 CBA/N B cells. One clone in 60 x 10^6 CBA/N spleen cells has been detected yielding a primary

TABLE 24

COMPARISON OF THE FREQUENCY OF PRIMARY TML-SPECIFIC B CELLS IN CBA/Ca AND CBA/N MICE

Donor	Treatment[a]	Total No. Cells Transferred ($\times 10^6$)	No. TML-Specific Clones Per 10^6 Cells Transferred[b]	No. TML-Specific Cells Per 10^6 Splenic B Cells[c]
CBA/Ca	$\bar{a}$-Thy 1 + C'	30	0.27	6.68
CBA/N	—	60	0.02	0.43
CBA/N	$\bar{a}$-Thy 1 + C'	225	0.00	0.00

a) Spleen cells were treated with T24/40.7 anti($\bar{a}$)-Thy 1 + HO-13.4 $\bar{a}$-Thy 1.2 + rabbit complement as outlined in Materials and Methods.

b) 10 to 25 x 10^6 $\bar{a}$-Thy 1 + C' treated CBA/N or CBA/Ca spleen cells and 30 x 10^6 untreated CBA/N spleens were injected into B10.BR recipients which had been previously primed with 1 x 10^3 AKD-TML organisms, i.v. 3 weeks before cell transfer. Stimulation of recipients and detection of clones by RIA were as described in Figure 7.

c) Calculated frequencies after homing efficiency and percent of B cells in the spleen were taken into account. The number of B cells in the spleens of CBA/Ca and CBA/N donor mice were taken to be 40% and 20%, respectively (Klinman et al., 1976; Scher et al., 1975).

TML-specific precursor frequency of 0.43 per 10^6 B cells. However, after analyzing 320 x 10^6 more CBA/N spleen cells, where as many as 60 x 10^6 cells were transferred to one recipient, no other TML-specific clones have been detected (data not shown). Thus, CBA/N spleens appear to contain either very few primary B cells or none at all.

Since CBA/N mice can generate a serum anti-TML antibody response, it is possible that the TML-specific precursors may exist in other lymphoid organs. Further, Eldridge et al. (1983) found that CBA/N mice may contain an Lyb-5^+ B-cell subset within the Peyer's patches since cells with mature characteristics were found that responded in vitro to both TD and TI-2 antigens. Therefore, it seemed likely that TML-specific precursors could be found in the Peyer's patches. The results presented in Table 25 suggest that this is not the case. As shown above, TML-specific precursors exist with equal frequency in the spleen and the Peyer's patches of CBA/Ca mice. In contrast, precursors are not detected in CBA/N Peyer's patches.

The fact that a serum response in CBA/N mice can be detected, yet primary B-cell precursors can not, suggests that the TML-specific precursors in these mice, like PC-specific precursors (Kenny et al., 1983), must undergo further differentiation before they can secrete antibody. If this is true, then memory anti-TML precursors should be detectable. Table 26 shows that CBA/N donor spleen cells transferred 6 weeks after immunization with 1 x 10^8 AKD-TML

TABLE 25

FREQUENCIES OF PRIMARY TML-SPECIFIC B CELLS IN THE SPLEEN AND PEYER'S PATCHES OF CBA/Ca AND CBA/N MICE

Donor	Cell Source	Total Number of Cells Transferred (X 10^6)	No. TML-Specific Clones per 10^6 Cells Transferred[a]
CBA/Ca	Spleen	80	0.19
CBA/N	Spleen	80	0.00
CBA/Ca	Peyer's Patches	55	0.24
CBA/N	Peyer's Patches	35	0.00

a) 40 x 10^6 donor spleen cells or 5-20 x 10^6 donor Peyer's patches cells were injected into AKD-TML primed B10.BR recipients. Priming and stimulation of recipients and detection of clones by RIA were as described in Figure 7.

TABLE 26

SECONDARY AND TERTIARY TML-SPECIFIC

PRECURSOR FREQUENCY IN CBA/Ca AND CBA/N MICE

Source of Cells	Donor Priming With AKD-TML	Total No. Cells Transferred	No. TML-Specific Clones Per 10^6 Spleen Cells[b] Transferred	No. TML-Specific Cells Per 10^6 Splenic B Cells[c]
CBA/Ca	-6 weeks	45×10^6	1.96 ± 0.12	122.5 ± 7.3
CBA/N	-6 weeks	55×10^6	0	0
CBA/Ca	-6 weeks, -2 weeks	40×10^6	2.85 ± 0.43	178.1 ± 27.0
CBA/N	-6 weeks, -2 weeks	310×10^6	0.12 ± 0.01	15.4 ± 1.8

a) CBA/N and CBA/Ca mice were immunized, i.v., with 1×10^8 AKD-TML organisms in saline either once, 6 weeks before or twice, 6 weeks and 2 weeks before using as donors.

b) 5×10^6 CBA/Ca or 5-20 $\times 10^6$ CBA/N donor spleen cells were injected into AKD-TML primed recipients. Priming and stimulation of recipients and detection of clones by RIA were as described in Figure 7. Data are presented as mean frequency $\pm$ 2 standard errors.

c) Calculated frequencies after homing efficiency and percent of B cells in the spleen were taken into account. The number of B cells in the spleens of CBA/Ca and CBA/N donor mice were taken to be 40% and 20%, respectively (Klinman *et al.*, 1976; Scher *et al.*, 1975).

organisms still do not give rise to detectable clones after secondary challenge. On the other hand, the secondary precursor frequency for CBA/Ca control spleen cells is 1.96 per 10^6 spleen cells, a 13-fold increase over the primary precursor frequency (see Table 11). However, CBA/N donor spleen cells which have been immunized once, 6 weeks before, then again 2 weeks before cell transfer with 1 x 10^8 AKD-TML organisms give rise to TML-specific precursors with a frequency of 0.12 per 10^6 spleen cells after tertiary challenge. This frequency is 23-fold lower than the frequency obtained for tertiary TML-specific precursors in CBA/Ca controls (compare lines 3 and 4, Table 26) but markedly similar to the primary precursor frequency in immunologically normal CBA/Ca mice (see Table 11).

Since CBA/N mice do not contain splenic Lyb-5^+ B cells, it appears, as suggested by Kenny et al. (1983) for the PC-specific response in the splenic focus system, that Lyb-5^- TML-specific B cells may need to differentiate into memory cells before they can mature into antibody-secreting cells. It is not clear, at this point, if the inability to detect primary anti-TML precursors in CBA/N mice is due to the failure of primary Lyb-5^- TML-specific B cells to respond in the splenic focus system or whether it is simply due to suboptimal conditions for stimulation of these B cells. This latter possibility seems unlikely because conditions for stimulation of DNP-specific precursors in

immune-defective and normal mice have been shown to be identical (Metcalf et al., 1980). In all experiments with CBA/N mice, CBA/Ca spleen cells were analyzed in parallel as a positive control.

3. Isotype analysis of monoclonal anti-TML-specific antibodies

The isotype profile of 46 tertiary TML-specific clones from CBA/N mice is shown in Table 27. In contrast to either the primary or memory CBA/Ca response, the majority of the CBA/N clones secrete IgG2 anti-TML antibody. Since CBA/N mice have low levels of serum IgG3 (Amsbaugh et al., 1972; Perlmutter et al., 1979) and are unresponsive to antigens which stimulate IgG3 production (Slack et al., 1980), it is not surprising that none of the CBA/N-derived clones secrete the IgG3 subclass. In addition, only a small proportion of these clones secrete either IgA or IgM anti-TML antibodies. These results indicate that, although the tertiary TML-specific precursor frequency in CBA/N mice is similar to the primary frequency in CBA/Ca mice, the distribution of the isotypes secreted from such clones is distinctly different. Further, although the CBA/N TML-specific clones have been generated after a tertiary challenge with AKD-TML, their isotype profile is also markedly different from that of CBA/Ca memory anti-TML clones.

The number of different isotypes secreted by

TABLE 27

PROPORTION OF CLONES FROM TERTIARY TML-SPECIFIC CBA/N B CELLS SECRETING PARTICULAR ISOTYPES

Donor Cells[a]	No. Clones Analyzed	% of Clones Secreting:[b]				
		IgM	IgG3	IgG1	IgG2	IgA
Primed CBA/N	46	8.7	0.0	28.3	73.9	13.0
Unprimed CBA/Ca	158	75.3	9.5	3.8	22.2	23.4
Primed CBA/Ca	208	71.6	23.1	35.6	57.7	44.7

a) Primed CBA/N and CBA/Ca donors were immunized as described in Table 26. TML-specific clones from primed CBA/N and CBA/Ca and unprimed CBA/Ca spleens were obtained from fragment cultures derived from AKD-TML primed AKR or B10.BR recipeints.

b) Anti-TML clones were analyzed for the isotype produced by RIA against whole TML organisms using rabbit anti-mouse heavy chain specific sera. Data from unprimed and primed CBA/Ca donor cells are repeated from Tables 12 and 15, respectively.

individual clones derived from CBA/N mice is compared to the number secreted from CBA/Ca primary and memory TML-specific foci in Table 28. Much like the CBA/Ca primary anti-TML clones, most CBA/N clones secrete only 1 isotype with a few secreting 2 or 3 isotypes. However, a closer examination of the complexity of isotype expression in anti-TML antibody from primary CBA/Ca and tertiary CBA/N TML-specific foci (compare Table 14 with Table 29) indicates subtle differences between these two B cell repertoires not revealed in Table 28. Table 29 shows the percent of CBA/N fragments which secrete anti-TML antibody of a particular isotype either alone or in combination with 1 to 4 additional isotypes. One difference, aside from the lack of IgG3 secreting clones, is that more than half of the CBA/N IgG1 anti-TML antibodies are secreted in the absence of other isotypes and the remainder of the clones which secrete IgG1 antibodies do so with only 1 additional isotype. Conversely, CBA/Ca primary IgG1 anti-TML antibodies tend to be secreted with most of the isotypes (see Table 14, line 3). A third difference is that the predominant isotype secreted by CBA/N clones is IgG2. Moreover, the majority of these clones secrete solely IgG2 anti-TML antibody. CBA/Ca primary clones, on the other hand, secrete IgG2 antibodies with at least 1 other isotype (Table 14, line 4). Although CBA/Ca primary clones are found to secrete 3 and 4 isotypes, it is likely that upon examination of additional CBA/N clones, the proportion

TABLE 28

NUMBER OF ISOTYPES SECRETED BY CLONES FROM TERTIARY TML-SPECIFIC CBA/N B CELLS

Donor Cells[a]	No. Clones Analyzed	% of Individual Clones Secreting the Following No. of Different Isotypes				
		1	2	3	4	5
Primed CBA/N	46	80.4	15.2	4.3	0	0
Unprimed CBA/Ca	158	72.2	21.5	5.7	0.6	0
Primed CBA/Ca	208	27.4	34.1	21.6	13.9	4.3

a) Primed CBA/N and CBA/Ca donors were immunized as described in Table 26. TML-specific clones from primed CBA/N and CBA/Ca and unprimed CBA/Ca spleens were obtained from fragment cultures derived from AKD-TML primed AKR or B10.BR recipients. Data from unprimed CBA/Ca donor cells are repeated from Table 13.

TABLE 29

ANALYSIS OF THE NUMBER OF ISOTYPES PRODUCED IN FRAGMENTS SECRETING GIVEN ISOTYPES OF ANTI-TML ANTIBODY [a]

Fragments Secreting the Following Isotype	No. of Clones	% of Individual Clones Secreting The following No. of Isotypes					$\bar{X}$ No. Isotypes Per Fragment
		1	2	3	4	5	
IgM	4	50.0	0	50.0	0	0	2.00
IgG3	0	0	0	0	0	0	0
IgG1	13	53.8	46.2	0	0	0	1.46
IgG2	34	76.5	23.5	0	0	0	1.24
IgA	6	33.3	66.7	0	0	0	1.67

a) TML-specific clones from primed CBA/N spleen cells were obtained from fragment cultures derived from AKD-TML primed AKR and B10.BR recipients. Total number of clones analyzed = 46.

secreting greater than 2 isotypes will approximate that of the CBA/Ca primary response.

As mentioned above, even after three immunizations, the isotype profile of CBA/N TML-specific clones differs drastically from that of CBA/Ca memory clones. Comparing Figure 16 to Figure 15 clearly illustrates these differences. These Figures indicate the percent of those primed CBA/N and primed CBA/Ca fragments, respectively, which secrete anti-TML antibody of a particular isotype (column A), that also secrete anti-TML antibody of each additional isotype (column B). Figure 15 shows that CBA/Ca memory TML-specific B cells have undergone considerable differentiation after 2 and 3 immunizations. Indeed, the majority of the clones secrete each isotype in combination with most of the other isotypes to a much higher degree than the CBA/Ca primary clones (compare Figures 10 and 15). In contrast, the majority of CBA/N clones which secrete each isotype, secrete only that isotype. The paucity of IgM anti-TML antibodies suggests that these TML-specific precursors have, indeed, differentiated into memory cells. However, the switching pathway appears to be different from that of CBA/Ca memory TML-specific B-cell precursors.

4. Fine specificity analysis of TML-specific B cells in CBA/N spleens

Previous studies have shown that bacterial carbohydrates stimulate antibody responses predominantly of

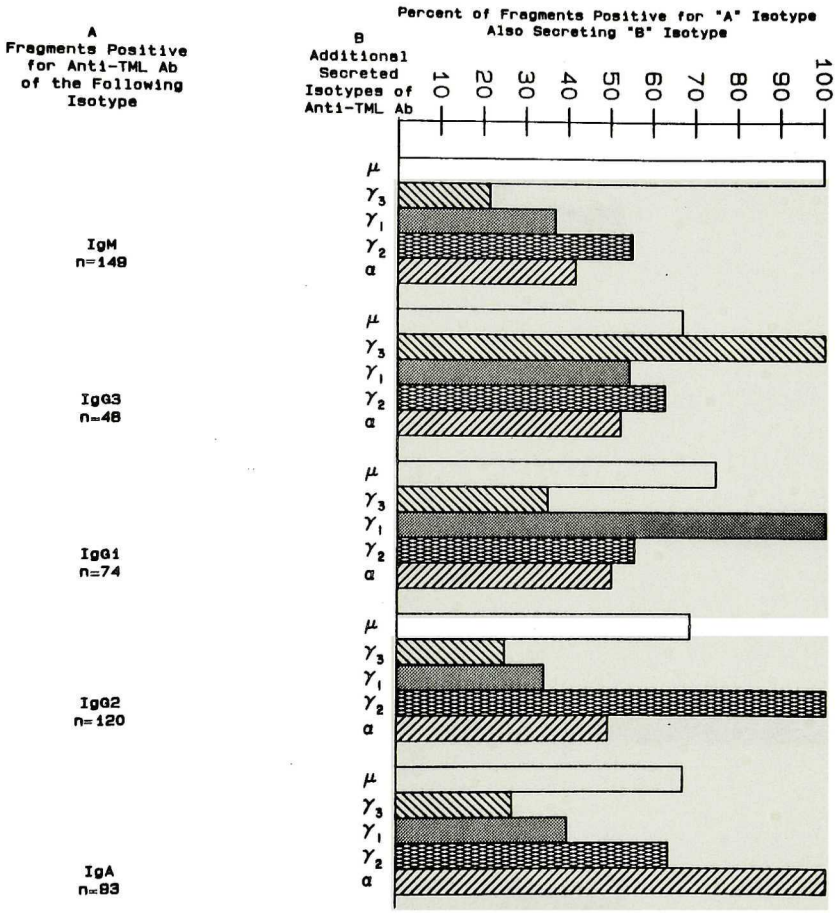

Figure 15. Analysis of anti-TML antibody (Ab) isotype co-expression by memory CBA/Ca B-cell clones. Primed CBA/Ca donors were immunized as described in Table 26. TML-specific clones from primed CBA/Ca spleens were obtained from fragment cultures derived from AKD-TML primed AKR or B10.BR recipients. The isotype(s) of the antibody produced by memory B-cell clones was determined by RIA using whole TML organisms as the immunoadsorbent and rabbit anti-mouse heavy chain class-specific sera. The total number of clones analyzed = 208.

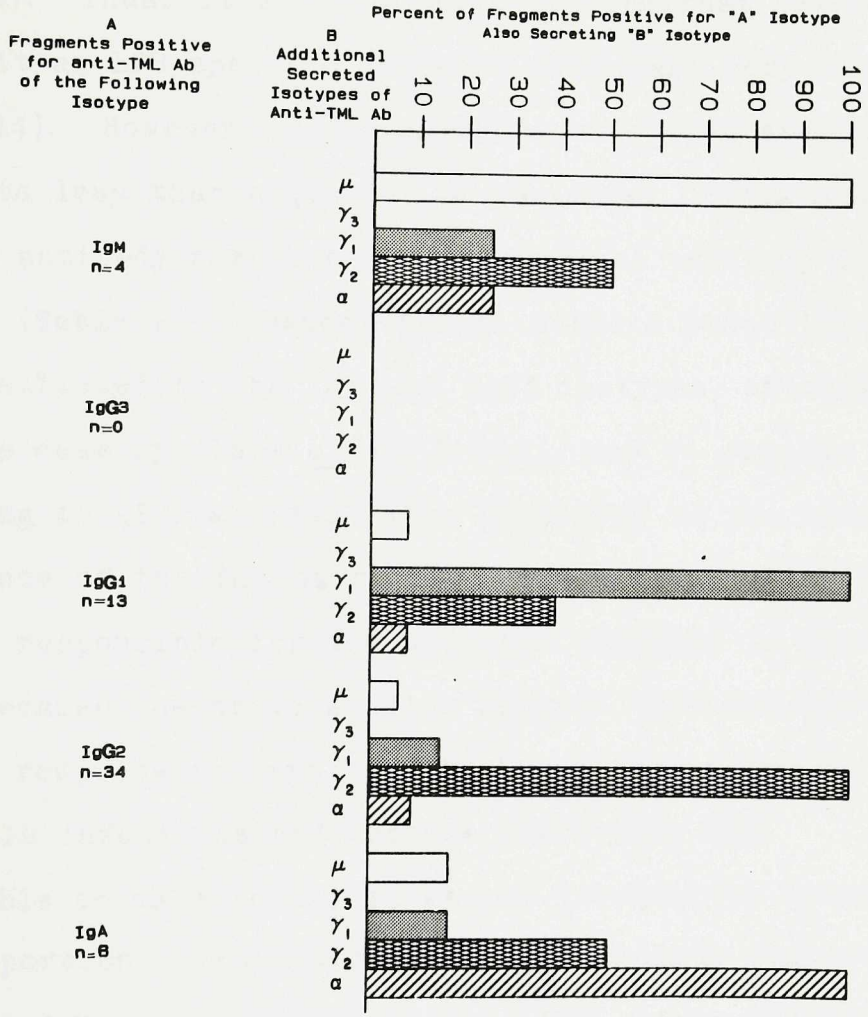

Figure 16. Analysis of anti-TML antibody (Ab) isotype co-expression by tertiary CBA/N B-cell clones. Primed CBA/N donors were immunized as described in Table 26. TML-specific clones from primed CBA/N spleens were obtained from fragment cultures derived from AKD-TML primed AKR or B10.BR recipients. The isotype(s) of the antibody produced by tertiary B-cell clones was determined by RIA using whole TML organisms as the immunoadsorbent and rabbit anti-mouse heavy chain class-specific sera. The total number of clones analyzed = 46.

the IgM and IgG3 isotypes (Perlmutter et al., 1978) and that CBA/N mice are unresponsive to such antigens (Slack et al., 1980). Thus, it is surprising to find that CBA/N mice can elicit an LPS-specific anti-TML serum antibody response (Figure 14). However, as shown above, the IgG3 subclass represents less than a quarter of the total CBA/Ca memory anti-TML antibody response which is almost entirely LPS-specific (Table 27). Hence, B-cell subsets other than those restricted to the IgM and IgG3 isotypes, as suggested to be the case by Slack et al. (1980), may be capable of responding to LPS, at least when presented to the host on the surface of the TML bacterium. These subsets, then, are probably responsible for the anti-LPS response in CBA/N mice. Because the above studies suggest that the LPS-specific response is important in the memory response to salmonella infections and because CBA/N mice are susceptible to salmonella, it was of interest to determine what proportion of the total anti-TML antibody response is contributed by clones specific for LPS. Table 30 shows that 69.6% of the 46 CBA/N TML-specific clones reacted with LPS in an RIA. In addition, none of these clones reacted with S. pneumoniae (data not shown). Therefore, although the frequency of TML-specific precursors is similar in unprimed CBA/Ca mice and primed CBA/N mice, the proportion of anti-LPS antibodies is over two-fold lower in unprimed CBA/Ca mice and 22% of the anti-TML response in these mice

TABLE 30

LPS SPECIFICITY OF TML-POSITIVE

CLONES FROM TERTIARY CBA/N SPLEEN CELLS

Donor Cells[a]	No. of Clones Analyzed	No. TML-Specific Clones Per 10^6 Cells Transferred[b]	% of Anti-TML Antibody Clones Reactive With TML-LPS[c]
Primed CBA/N	46	0.12	69.6
Unprimed CBA/Ca	420	0.15	28.6
Primed CBA/Ca	208	2.85	96.6

a) Primed CBA/N and CBA/Ca donors were immunized as described in Table 26.

b) Frequencies for primed CBA/N and CBA/Ca cells are from Table 26. Frequency for unprimed CBA/Ca spleen cells is from Table 11.

c) Anti-TML positive clones in fragment cultures derived from recipients primed with AKD-TML were assayed in a solid-phase RIA with either TML-LPS or Streptococcus pneumoniae as the immunoadsorbent.

is reactive with S. pneumoniae. The frequency of LPS-specific clones in tertiary primed CBA/N mice has an intermediate value between unprimed and primed CBA/Ca mice.

Recent studies by Kenny et al. (1983) demonstrated that primary PC-specific precursors from immune-defective mice were not detectable in the splenic focus system, but secondary precursors were detectable at a frequency 10-fold lower than the secondary response observed for normal controls. Furthermore, Clough et al. (1981) reported that the frequency of PC-specific precursors was similar in immune-defective and normal mice after 3 immunizations. Therefore, it is possible that the frequency of quaternary TML-specific precursors from CBA/N mice will approach that found in CBA/Ca mice. In addition, the proportion of the response that is LPS-specific would also be expected to approximate that observed in normal mice.

The 31 TML-specific clones from CBA/N mice shown to be reactive with LPS were analyzed further for reactivity with a series of rough S. typhimurium mutants. It was anticipated that discrete patterns of reactivity could possibly elucidate the susceptibility of CBA/N mice to S. typhimurium. The results of such an analysis are compared to the responses of unprimed and primed CBA/Ca mice in Table 31. The results indicate that 67.7% of the TML-LPS-specific precursors from CBA/N mice does not react with any of the O-antigen-deficient rough mutants tested. Four of the clones recognize a site somewhere within the LPS outer

TABLE 31

FINE SPECIFICITY OF ANTI-LPS ANTIBODIES FROM CBA/N TML-SPECIFIC CLONES

Source of Cells	Total No. Clones Analyzed	Immuno-adsorbent	% Total LPS+ Clones with Following Reactivity Pattern[a]					
		LPS	+	+	+	+	+	+
		Ra	−	+	+	+	+	+
		Rc	−	−	+	+	+	+
		Rd1	−	−	−	+	+	+
		Rd2	−	−	−	−	+	+
		Re	−	−	−	−	−	+
Primed CBA/N	31		67.7	12.9	0	3.2	0	16.1
Unprimed CBA/Ca	104		72.1	0	0	1.9	0	26.0
Primed CBA/Ca	199		43.7	2.0	1.5	3.0	5.5	44.2

a) Anti-TML positive clones reactive with LPS were assayed using whole S. typhimurium rough mutant organisms as immunoadsorbents. Data from unprimed and primed CBA/Ca donor cells are repeated from Table 19.

core polysaccharide region, but not beyond the galactose II molecule (see Figure 12). One clone recognizes a site on the heptose II molecule of the inner core region. Finally, only 5 clones react with all the tested rough mutants. These results are comparable to the reactivity pattern of primary TML-LPS-specific clones from CBA/Ca mice. Both responses similarly have considerable differences in reactivity patterns from that of primed CBA/Ca mice. This is more clearly illustrated in Figure 17. Whereas the majority of the CBA/N anti-LPS antibodies are directed against the O-antigen and only a small proportion is directed against the KDO or lipid A region, less than half of the CBA/Ca memory anti-LPS antibodies are O-antigen-specific and nearly half are KDO or lipid A-specific.

These results suggest that Lyb-5^+ B cells produce both anti-O-antigen and anti-lipid A antibodies. While Lyb-5^- B cells are capable of producing anti-LPS antibodies, these antibodies are predominantly O-antigen-specific. Therefore, the anti-O-antigen antibodies from primed CBA/Ca mice presumably arise from both Lyb-5^- and Lyb-5^+ TML-specific B cells, whereas, anti-lipid A antibodies apparently arise from only Lyb-5^+ B cells. An analysis of more CBA/N clones may clarify the differences in LPS reactivity between the three responses. For example, this interpretation of the results would predict that the proportion of O-antigen-specific clones would

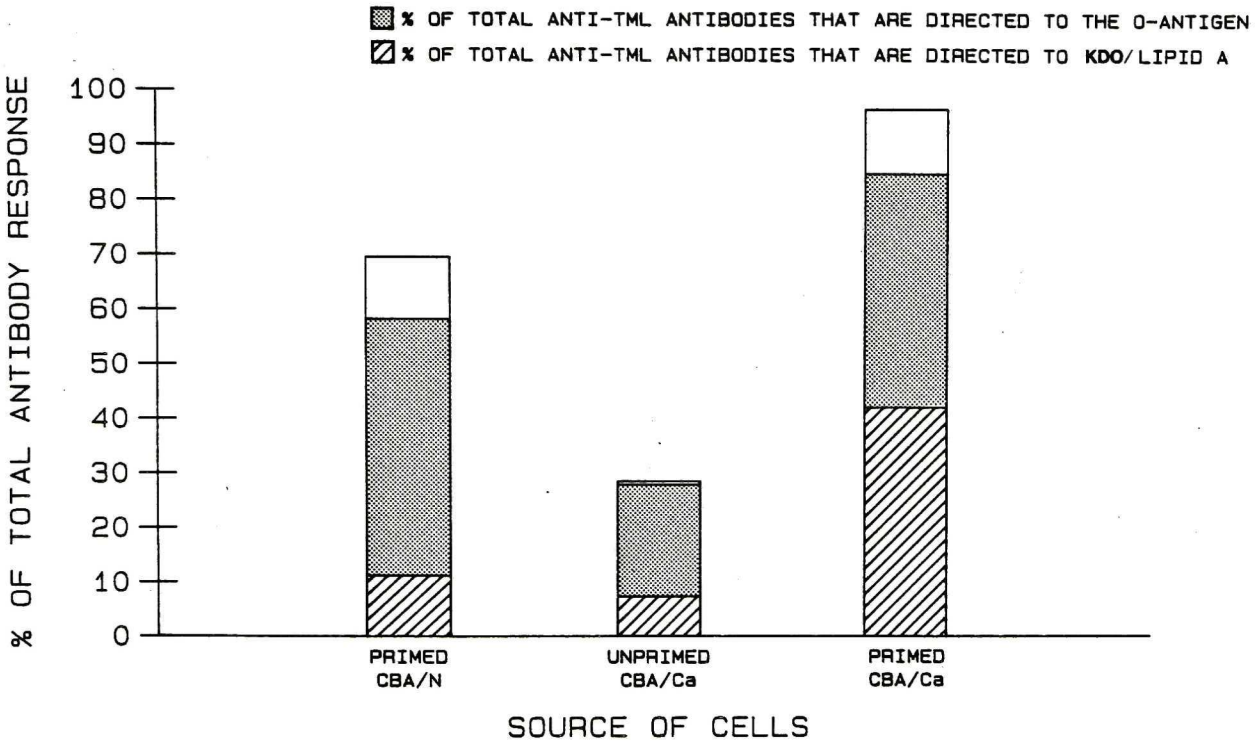

Figure 17. TML-specific clones from primed CBA/N mice and from unprimed and primed CBA/Ca mice were compared by RIA using LPS and a series of S. typhimurium rough mutants as immunoadsorbents. The entire bar represents the percent of the total anti-TML antibody response that are reactive with LPS. The open areas of the bars represent the percent of clones which react with LPS-core determinants.

increase and the proportion of lipid A-specific clones would decrease for tertiary LPS-specific CBA/N B cells. Additionally, treatment of primed CBA/Ca spleen cells with anti-Lyb-5 and C' should effectively diminish the anti-lipid A component of the memory anti-TML antibody response if this hypothesis is correct.

Lyb-5^- B cells also appear to respond to antigenic determinants other than LPS (Table 30). Moreover, since primary TML-specific precursors from CBA/N mice do not respond in the splenic focus system and because over 70% of the primary precursors from CBA/Ca mice do not react with LPS, it is assumed Lyb-5^+ B cells also produce antibodies against other salmonellae components.[3] Table 32 compares the reactivity of LPS-negative TML-specific clones from primed CBA/N mice and unprimed and primed CBA/Ca mice against POL, porin, and lipid A. However, there are too few clones from primed CBA/N and CBA/Ca mice to gain any discriminative information with regard to the reactivity of these particular determinants. Nevertheless, Figure 18 shows the percent of the total TML-specific B-cell clones

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[3] It has yet to be determined if, in fact, the primary Lyb-5^- B-cell subset from CBA/Ca mice fail to respond in the splenic focus system. This can be determined by analyzing the response of unprimed CBA/Ca spleen cells after treatment with anti-Lyb-5 and C'. This type of analysis is necessary to define the limitations of this kind of assessment of the B-cell repertoire responsive to TML. It is possible that the preceding analysis of the primary TML-specific B-cell repertoire in CBA/Ca mice may be skewed to the Lyb-5^+ B-cell subset.

TABLE 32

REACTIVITY OF LPS-NEGATIVE TML-SPECIFIC CLONES FROM CBA/N MICE

Source of Cells	Total No. Clones Analyzed	Immuno-adsorbent	% of Total LPS⁻ Clones with the following Reactivity Pattern[a]							
		POL	+	-	-	+	+	-	+	-
		Porin	-	+	-	+	-	+	+	-
		Lipid A	-	-	+	-	+	+	+	-
Primed CBA/N	15		13.3	0	0	0	0	0	6.7	80.0
Unprimed CBA/Ca	187		7.5	8.0	2.7	5.3	2.7	1.1	15.5	57.2
Primed CBA/Ca	5		40.0	0	0	40.0	0	0	0	20.0

a) TML-specific clones which did not react with LPS were re-assayed by RIA using POL, porin protein, or lipid A as the immunoadsorbent according to the procedure described in Materials and Methods. Data from unprimed and primed CBA/Ca donor cells are repeated from Table 20.

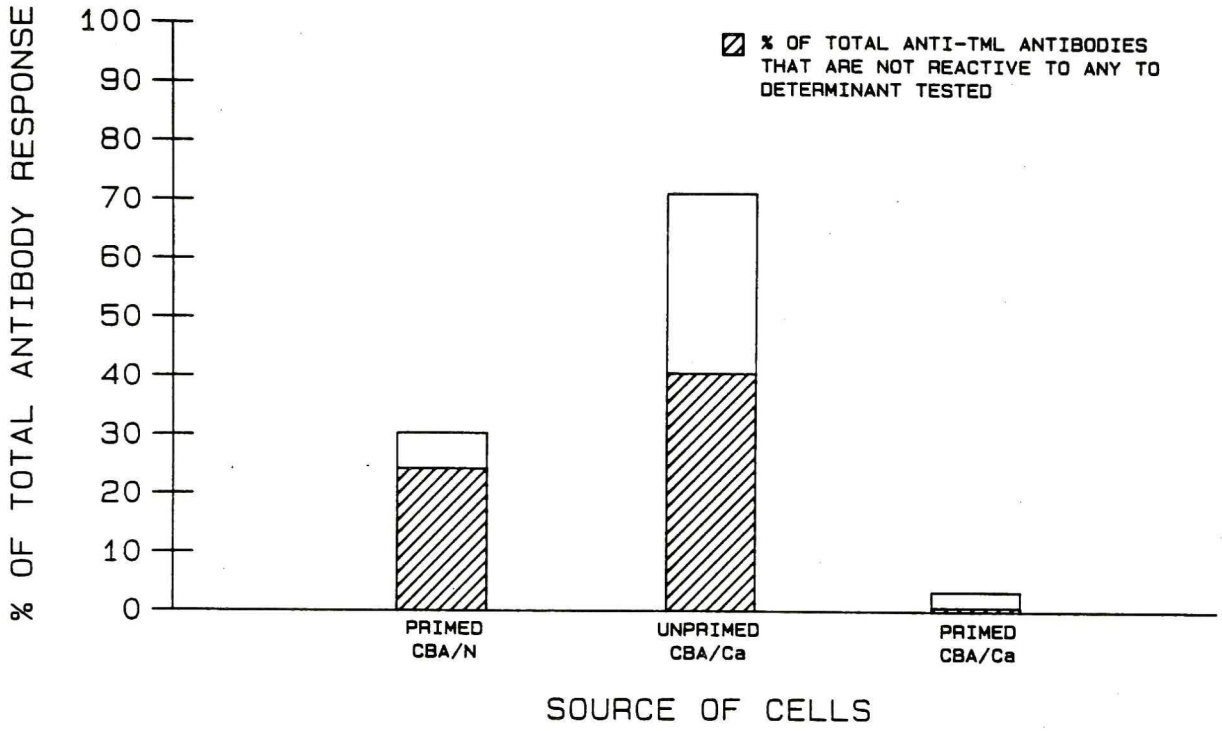

Figure 18. TML-specific clones from primed CBA/N mice and from unprimed and primed CBA/Ca mice which did not react with LPS were compared by RIA using POL, porin protein, and lipid A as immunoadsorbents. The entire bar represents the percent of the total anti-TML antibody response that are not reactive with LPS. The open areas of the bars represent the percent of clones which react with POL, porin protein, and/or lipid A.

from primed CBA/N mice and unprimed and primed CBA/Ca mice which do not react with LPS (30.4% in primed CBA/N mice; 71.4% in unprimed CBA/Ca mice; 3.4% in primed CBA/Ca mice). It is interesting to note that the majority of LPS-negative anti-TML antibodies from primed CBA/N and unprimed CBA/Ca mice are not directed against any of the surface antigens tested. Figure 18 indicates that 30.6% of the total TML-specific clones derived from unprimed CBA/CA mice reacted with POL, porin protein, and/or lipid A. However, almost 41% of the primary TML-specific response (and, possibly, the Lyb-5^+ subset) in CBA/Ca mice is not directed against any of the surface antigens tested. Although the majority of LPS-negative TML-specific B-cell clones from primed CBA/N mice is in fact not directed against any of the tested antigenic determinants (Figure 18), these clones represent less than a quarter (24%) of the total tertiary (Lyb-5^- subset) response in these mice. Whether reactivity of these unclassified anti-TML antibodies distinguishes CBA/N responses from CBA/Ca responses remains to be determined.

IV. Discussion

In this thesis, the *in vitro* splenic focus system has been modified to examine the responses of immunologically normal, salmonella-resistant and immunologically-defective, salmonella-susceptible precursor B cells to *S. typhimurium* strain TML. In conjunction with a sensitive radioimmunoassay (Metcalf and O'Brien, 1981), these studies provide not only an estimate of the TML-specific B-cell precursor frequency, but also a characterization and comparison of the isotype(s) and fine specificity of the antibody product of the clonal progeny of individual, TML-stimulated B cells in normal and genetically-susceptible, antibody-defective mice.

A. Analysis of the Splenic TML-Specific B-Cell Repertoire in CBA/Ca Mice

The mean frequency of TML-specific B cells in non-immune, CBA/Ca mice of 9.6 per 10^6 splenic B cells, is extremely small when compared to the frequency of DNP-responsive cells which is 200 per 10^6 B cells (Klinman *et al.*, 1976). In addition, this observed frequency is 15- to 20-fold lower than the frequency observed for several different chemically defined haptenic determinants (see Sigal and Klinman, 1978). However, the frequencies of B cells responsive to other natural determinants are also several orders of magnitude lower than the frequency to the chemically defined molecules. Indeed, Cancro *et al.* (1978)

found that the frequency of influenza HA-specific B cells is, on the average, 13 per 10^6 B cells. The frequency of PC-responsive cells was determined to be 19 per 10^6 B cells (Sigal et al., 1975) and that of anti-cholera toxoid antibody-producing cells was <1 per 10^6 B cells (Fuhrman and Cebra, 1981; Cebra et al., 1982). Cancro et al. (1978) sought to explain these observations. These investigators contended that complex antigens, such as bacteria and viruses, are composed of antigenic determinants which may be very similar to self antigens, and, as a consequence, only a small number of determinants are recognized as foreign. Thus, the frequency of B cells specific for bacteria and viruses would be low. In contrast, simple haptenic determinants are clearly non-self structures which allow the stimulation of multiple B-cell precursors. This hypothesis is attractive; however, it does not explain the low frequency of PC-specific precursors. PC, although found naturally on the cell walls of S. pneumoniae, is a simple haptenic determinant. A more likely interpretation of these studies, which would also explain responses to antigens like PC, is that clonotypes responsive to determinants on these natural antigens cross-react with antigens abundantly present in nature. In this way, most clonotypes would be tolerized during ontogeny (Metcalf and Klinman, 1976; Metcalf et al., 1979; Teale and Klinman, 1980). Metcalf and Klinman (1976) provided

evidence in the splenic focus system that developing B cells mature through a stage in which they are extremely susceptible to tolerance induction. Furthermore, their results suggested that the specific interaction of B-cell antigen receptors with multivalent antigens (such as bacteria) is tolerogenic to neonatal (immature) B cells unless antigen is concomitantly recognized by primed T cells. Conversely, mature B cells are not tolerized by this type of interaction. Hence, most S. typhimurium-specific clonotypes in developing B cells would be tolerized rather than stimulated by interaction with cross-reactive normal gut flora since primed T cells would not as yet be present. Only a limited subset of clonotypes, which recognize determinants distinct from environmental antigens, would be available to respond upon deliberate primary stimulation. By this argument, the frequency of primary B cells responsive to natural antigens would be expected to be low. Furthermore, secondary challenge with natural antigens would provide sufficient helper T cells for stimulation of newly generated B cells emerging from the bone marrow (Metcalf and Klinman, 1976, 1977). Therefore, the secondary TML-specific repertoire would be expected to include those tolerizable B-cell subsets which would be missing from the primary repertoire as well as those B-cell subsets which would not be tolerized. Thus, the precursor frequency would be expected to increase after secondary challenge. By contrast, clonotypes specific for

chemically defined determinants which are not found in nature would not be tolerized. Therefore, upon deliberate primary immunization, multiple B-cell precursors for these synthetic antigens would be stimulated and the overall frequency would appear larger than the frequency for natural antigens. Hence, relative to the increase in precursor B-cell frequency which occurs after challenge with natural antigens, the increase in B-cell frequency after secondary challenge with synthetic antigens would seem less significant. Indeed, after immunization, the frequency of TML-specific B cells increases from 1 in 1 x 10^5 splenic B cells to 1 in 5.6 - 8.1 x 10^3 splenic B cells whereas the frequency of DNP-specific B cells increases from only 1 in 5 x 10^3 to 1 in 1.3 x 10^3 splenic B cells (Klinman and Press, 1975).

Recent studies by Thompson et al. (1982, 1983) have extended the initial observations of Klinman and Press (1975) on the regulation of B-cell repertoire formation. These investigators examined the kinetics and clonal composition (reactivity patterns) of the PR8 HA-specific repertoire in BALB/c mice at 1 and 2 weeks of age in the splenic focus system. Their findings indicated that the clonal composition of the primary HA-specific B-cell pool changes rapidly and regularly during the first two weeks of life. However, when PR8 was administered to mice at 1 week of age, the reactivity patterns observed in these immunized

mice at 2 weeks of age, had not markedly changed from those observed in normal 1 week old mice. Many clonotypes that normally arose between 1 and 2 weeks of age in normal mice failed to be significantly expressed. Furthermore, several clonotypes that were normally only transiently expressed at 1 week of age were preferentially expanded and preserved within the immunized HA-specific B-cell pool. In addition, the repertoires of adult mice that had either been chronically or acutely immunized since the first week of life were very similar to the primary HA-specific repertoire at 1 week of age. Their results demonstrated that immunized individuals displayed repertoires most similar to that repertoire which was characteristic of the age when immunization first occurred. Therefore, antigen exposure appears to perturb the normal turnover in clonotype composition of the emerging B-cell repertoire. Their results suggested this may occur by the preservation of clones that are otherwise transiently expressed, and by the prevention of the appearance of certain other HA-specific clones. Based on these studies, it would be predicted that, early in development, many clonotypes emerging within the salmonella-specific B-cell repertoire would be preserved upon contact with normal gut flora, while other clonotypes would be deleted. Furthermore, if these animals had no prior contact with cross-reactive environmental antigens, the TML-specific B-cell repertoire expressed by adult mice may be somewhat different.

Examination of primary TML-specific precursors in germ-free mice should determine if environmental antigen exposure leads to tolerance of some salmonella-specific primary B-cell precursors. Indeed, the full potential of TML-responsive clones should be expressed in these mice and, thus, a higher precursor frequency would be observed. However, primary clonotype expression in germ-free mice will also be under a state of flux, that is, distinct clonotype precursor cells will enter the B-cell pool, clonally expand, and then exit from the pool at different points in time (Klinman and Press, 1975). Thus, even if the overall frequency increases over that in conventional mice, the expression of particular clonotypes may not change.

Cancro and Thompson also suggested that active mechanisms play a role in repertoire development by selectively fixing particular clonotypes after antigen exposure. Their results from in vivo T cell transfer experiments demonstrated primary HA-specific humoral responses to both the homologous and a heterologous influenza virus strain were completely suppressed by T cells from mice chronically primed with virus since birth. Further, secondary humoral responses were resistant to the suppressive effects. Suppression was abolished by treating the donor T cells with anti-Lyt-2 and C' or by irradiation of the T-cell inoculum. Similar regulatory mechanisms may

affect the establishment of the TML-specific B-cell repertoire. Thus, it is possible that exposure to cross-reactive antigens may induce T cells which suppress particular TML-reactive clones and prevent their clonal expansion.

Isotype analysis of TML-specific clones showed that natural and synthetic antigen B-cell responses were distinct in yet another way (Press and Klinman 1973c; Teale et al., 1981). Whereas the predominant isotypes secreted by TML-specific B-cell clones was IgM, IgG2 and IgA, classic synthetic TD antigens, for example, DNP-Hy, stimulate predominantly IgG1 secretion (Press and Klinman 1973c; Slack et al., 1980; Teale et al., 1981). One may have expected that anti-TML antibodies would follow the same isotypic pattern as DNP-Hy (with respect to IgG1 production) since the overall response to S. typhimurium appears to be T-cell dependent. Indeed, recent studies by this laboratory (O'Brien et al., 1981) reported that: 1) CBA/Ca nu/nu mice are susceptible to TML infection, unlike euthymic mice of the CBA/Ca background strain; and 2) CBA/Ca nu/nu mice are unable to produce IgM or IgG anti-S. typhimurium antibodies when challenged with AKD-TML. Furthermore, anti-TML antibody-producing clones in the splenic focus system are undetectable in fragments derived from unprimed recipients (this thesis; E.S. Metcalf, unpublished observation). Thus, it appears that the response to TML is T-dependent and that stimulation of

primary TML-specific precursors in the splenic focus system is dependent upon the presence of antigen-primed recipient T cells. However, the isotype profile of TML-specific B-cells is distinct from classical TD antigens such as DNP-Hy. It is important to note that the isotype profile is also different from the distribution typically reported for either TI-1 or TI-2 antigens (Slack et al., 1980; Mongini et al., 1982, 1983). TI-1 antigens generally stimulate equivalent levels of IgG3 and IgG2 subclasses whereas TI-2 antigens stimulate primarily the IgG3 subclass. The predominant IgG subclass stimulated by TML, on the other hand, is IgG2 only.

If IgG1 production is indicative of a classic TD response, and if the response to S. typhimurium is truly TD, then it may be possible that recipient T helper cells are not sufficiently stimulated in the priming regimen utilized in these studies. The data which analyze the complexity of isotype expression in antibodies produced by primary TML-specific clones does support this possibility. Although many clones secreted anti-TML antibodies of 2 to 4 different isotypes, the majority of clones secreted predominantly IgM. This suggests that the recipient T cells have not been optimally stimulated to help switch immunoglobulin production from the IgM isotype to other isotypes. An even more comprehensive immunization schedule may increase T-cell priming which in turn may lead to an

increased IgG1 anti-TML antibody response and possibly to an increased TML-specific precursor frequency. However, even with three immunizations, no significant change in T-cell help was observed. It is also possible that the response to AKD-TML is not truly representative of the response to the viable bacterium. Acetone treatment of S. typhimurium may destroy or alter relevant antigenic determinants and, thus, skew the actual response. Although these concerns are particularly pertinent when assessing the B-cell repertoire responsive to a complex antigen, it is interesting that the observed low frequency and distinct isotype profile of these TML-specific clones are markedly similar to those of the influenza HA-specific clones (Cancro et al., 1978). The response to this complex viral antigen has been shown to be TD (Cancro et al., 1978; Pierce et al., 1978; Virelizier et al., 1974), yet only a small proportion of the HA-specific B-cell clones produce IgG1 (Cancro et al., 1978, Wylie and Klinman, 1981). Additionally, Wylie and Klinman (1981) demonstrated that the majority of HA-specific clones stimulated with an influenza-infected syngeneic cell line in splenic fragment cultures produced antibodies of the IgG2b and IgG2a subclasses. Like the response to purified influenza virus, only a small proportion of these antibodies were of the IgG1 subclass. In fact, these data may suggest the importance of IgG2 and IgA antibodies in pathogenic infections. Therefore, although much information has been

gained from the study of immune responses to simple haptenic determinants, it appears that such responses can not be generally applied to more biologically relevant antigens such as TML and influenza HA.

Though the distribution of isotypes differs between antibodies produced by primary TML-specific precursors and those produced by primary DNP-Hy responsive precursors, the patterns of expansion from primary to memory clones appear similar between these two responses in terms of heterogeneity in isotype expression. Indeed, the number of clones which secrete IgM in both memory responses does not increase appreciably from the primary response. However, both memory TML-specific and DNP-specific responses have increased numbers of clones which produce several different isotypes. The relative frequency of IgG1 secreting TML-specific clones increases over 9-fold in the memory response when compared to the primary response. Although the proportion of the memory clones which secretes IgG1 is much lower in the TML-specific response than it is in the DNP-specific response, this isotype increases significantly more than any other isotype. These results may indicate that IgG1 production is as important in memory anti-S. typhimurium antibody responses as it is in DNP-specific memory responses. Again, if T helper cells are not sufficiently stimulated during the priming regimen, then a more rigorous immunization protocol may prove to stimulate

comparable relative frequencies of each isotype between TML and DNP memory responses. However, if T cells were absolutely required in TML-specific responses, yet not sufficiently provided, one might not expect as large an increase in the number of antigen-specific B cells as was observed after TML antigen exposure.

Although responses to AKD-TML could be skewed, the following data suggest that this is not the case. The S. typhimurium antigens which elicit protective antibodies in mice have not, as yet, been identified. However, LPS is a major cell surface antigen in the murine host immune response to this infectious bacterium (Sonnenwirth, 1973). Metcalf and O'Brien (1981) have demonstrated that a major component of the anti-S. typhimurium antibodies in sera of mice immunized with live TML is directed against LPS. Furthermore, the $L.D._{50}$ of LPS O-antigen-deficient S. typhimurium strains (rough mutants) has been found to be 10,000- to 100,000-fold higher for mice than that of smooth parental strains (Germanier, 1970). These results indicate that rough mutants have lost their virulence and suggest that the O-polysaccharide chain of S. typhimurium LPS is a critical virulence factor. Hence, it is no surprise that the LPS-specific precursors are preferentially expanded upon secondary stimulation with AKD-TML. Immunization with AKD-TML reflects the apparent importance that anti-LPS antibody plays in the immune response to S. typhimurium infections. On the other hand, it is possible that the AKD

preparation may preferentially elicit an anti-LPS response. If so, then one would expect that the primary response would also reflect a preferential LPS response. Contrary to this prediction, less than 30% of the anti-TML antibodies produced by primary CBA/Ca clones are LPS-specific. Therefore, AKD-TML does appear to stimulate a true representation of the TML-specific B-cell repertoire or, at least, an approximation of the repertoire in terms of what is known about the role of LPS in the anti-S. typhimurium antibody response and in virulence (see Roantree, 1967; Germanier, 1970, 1972; Ornellas et al., 1970; Eisenstein, 1975; Kiefer et al., 1976; Svenson et al., 1979; Metcalf and O'Brien, 1981). Furthermore, the apparent selective expansion of clones directed towards one of the major salmonella cell surface determinants (LPS) is reminiscent of the antigen-dependent expansion of influenza-specific memory B-cell clones where an increase from 30% to greater than 80% of the anti-influenza antibodies were found reactive to HA, a major influenza virus determinant (Cancro et al., 1978). Thus, again, AKD-TML has provided patterns of stimulation similar to those of another infectious agent.

The majority of the anti-LPS antibodies derived from primary CBA/Ca TML-specific clones is directed against the O-antigen. Since the O-antigen is a critical virulence factor, these findings suggest that anti-O-antigen

antibodies may play an important role in the resistance to S. typhimurium infections. Interestingly, even though O-antigen-specific precursors are significantly expanded after antigen exposure (from 2 per 10^6 B cells to 50-75 per 10^6 B cells), these B cells make up less than half of the total CBA/Ca memory LPS-specific B-cell response. KDO/lipid A-specific precursors increase approximately 100-fold (from 0.7 per 10^6 B cells to 50-75 per 10^6 B cells) and comprise most of the remaining half of the memory LPS-specific response. Very little of the primary anti-LPS antibody response is directed against core polysaccharide determinants and, in turn, these clones do not compose a significant proportion of the memory response. It is possible that because the core region of the LPS molecule displays considerably less chemical and antigenic diversity between strains and species of gram-negative bacteria in comparison to O-antigenic determinants (Lüderitz et al., 1966), cross-reacting antigenic exposure may have tolerized the vast majority of clones reactive to these determinants. A second possible explanation for these findings is that core determinants may be concealed by the O-antigen polysaccharide and, as a consequence, stimulation of B-cell precursors specific for these determinants is prevented. Furthermore, the O-antigen, core polysaccharide or other cell wall components may conceal lipid A antigenic sites as well. Hence, LPS released from the cell wall (Collins, 1974) probably serves as the immunogen for lipid A-specific

B-cell clones. Ostensibly, core determinants can not stimulate B cells as effectively as the two ends of the LPS molecule. The results presented in this thesis suggest that antibodies to the core region of LPS are not important in the memory response to S. typhimurium infections but that both anti-O-antigen and anti-lipid A antibodies play a role. In contrast, studies by Germanier (1970) and Braude et al., (1977) have indicated that vaccination with rough mutants and passive transfer of anti-core polysaccharide serum could protect against a lethal challenge with a smooth strain. It is important to note, however, that in the first study (Germanier, 1970), protection was measured for only one week. A longer period of time is necessary to demonstrate the protective effects of antibody in a lethal infection since anti-S. typhimurium antibodies appear to be important late in infection (O'Brien et al., 1981). In the second study (Braude et al., 1977) anti-salmonella core antibody was tested for its cross-protective capacity for a 3 day period against a heterologous gram-negative bacillus. Thus, these studies remain inconclusive. In addition, Germanier and Furer (1971) showed that the galE rough mutant used to immunize mice in the former study (Germanier, 1970) was able to obtain galactose in vivo. Thus, this mutant was able to synthesize a smooth LPS molecule. Therefore, the protection afforded by this particular rough mutant was probably due to anti-O-antigen

antibodies rather then anti-core antibodies.

The results of Table 19 in this thesis suggest that O-specific and lipid A-specific precursors are important in resistance to salmonella, and are consistent with several other studies. Firstly, studies by Ornellas et al. (1970) suggested the importance of anti-O antibodies in increasing the $L.D._{50}$ of a virulent salmonella strain which expressed O-antigens 9 and 12 (O:9,12). These investigators pre-opsonized the virulent strain with hyperimmune serum raised against a homologous heat killed strain or against a heterologous (O:4,12) vaccine strain. Serum raised against the O:9,12 specificity increased the $L.D._{50}$ of this strain 1,000-fold. Conversely, the O:4,12 specific serum raised the $L.D._{50}$ only 10- to 100-fold. In addition, Kiefer et al. (1976) constructed an E. coli-S. typhimurium hybrid which expressed the S. typhimurium O-antigens 4, 5, 12. Mice immunized with this hybrid were significantly protected from lethal challenge with a live, virulent S. typhimurium (O:4,5,12) strain but not with the parental E. coli strain (O:8). Moreover, Svenson and Lindberg (1981) demonstrated that rabbit antiserum raised against S. typhimurium O:4,12-antigen-specific saccharide- BSA conjugates passively protected mice against a lethal infection with S. typhimurium (O:4,12) but not with S. enteritidis (O:9,12). Taken together, these studies support the importance of O-specific B-cell precursors in the immune response to salmonella.

The importance of anti-lipid A antibodies in resistance to salmonella is not as clearly demonstrated as anti-O antibodies. Since the lipid A region of the LPS molecule has been found to be structurally identical or similar in all Enterobacteriaceae (Lüderitz et al., 1971), most studies deal with the capacity of anti-lipid A antibodies to passively protect mice against heterologous challenge with gram-negative organisms. The results of such studies have yielded conflicting conclusions. Early studies by Chedid et al. (1968) suggested that antibody to S. typhimurium lipid A protected mice from lethal challenge infections with Klebsiella pneumoniae. However, this putative anti-lipid A serum was raised against whole cells of S. typhimurium Re mutants. Using purified S. minnesota lipid A-protein conjugates to elicit anti-lipid A antibodies in rabbits, Mullan et al. (1974) were unable to demonstrate passive protection against lethal E. coli challenge with this rabbit antiserum. Recently, chemical analyses of the lipid A moiety of the LPS molecule from different gram-negative bacteria demonstrated considerable species variations as well as significant minor heterogeneity (see Kotani et al., 1983; Rietschel et al., 1983). Consequently, cross protection may not be observed. Lipid A may only elicit protective antibodies against bacteria containing homologous lipid A chemical structures. Rietschel and Galanos (1977) have demonstrated an

involvement of anti-lipid A antibodies in the protection against the pyrogenic responses and against skin necrosis in the local Shwartzman reaction elicited in rabbits by either purified LPS or lipid A. Interestingly, antibody to O-antigens did not protect against the LPS-induced fever response. Thus, anti-lipid A antibodies appear to have a beneficial role in protection against gram-negative infections; however, the mechanism of this protection appears to be different from anti-O-antigen antibodies (Rietschel and Galanos, 1977). Therefore, the preferential expansion of O-antigen-specific and KDO/lipid A-specific B-cell subsets is consistent with the idea that anti-O-antigen and anti-lipid A antibodies may be important in the memory response to TML as suggested by the preceding studies. Together, these two antibody responses may play a synergistic role in the resistance and/or recovery from a virulent salmonella infection. Perhaps, anti-O-antigen antibodies are important as opsonins which ultimately lead to inhibition of bacterial multiplication and, perhaps, anti-KDO/lipid A antibodies are important in preventing endotoxin-mediated lethality. These mechanisms remain to be elucidated.

S. typhimurium contains a multiplicity of potential antigenic determinants. The LPS molecule alone is composed of chemically diverse determinants which, as the results herein suggest, can elicit antibodies with a wide array of specificities. Ostensibly, antibody to those determinants

which extend out from the outer membrane of the bacterium would comprise most of the TML-specific response. The results presented show that less than a third of the primary CBA/Ca TML-specific B-cell precursors are directed to LPS with even a smaller proportion directed to flagella or the 34K porin protein. All three of these antigens extend beyond the outer membrane (Shands, 1966; Davis, 1973; Nurminen et al., 1976; Kamio and Nikaido, 1977). Too few clones were available to predict the relative importance of these latter two antigens in the CBA/Ca memory response. Because of the presumed accessibility of flagella and porins to the environment, it might have been expected that a larger proportion of primary clones would react with POL or the porin protein. However, there are many other antigenic determinants which are exposed on S. typhimurium. Although they may not be major surface components, much of the primary response may be directed against these other determinants. Furthermore, field trials have shown that anti-flagella antibody titers significantly increase after immunization with AKD salmonella vaccines (see Levine and Hornick, 1983). Although only a small number of memory clones was examined, a selective expansion of POL-specific clones after antigen exposure is suggested which is consistent with the AKD vaccine field trials. Analysis of additional clones is needed to confirm this finding. Though AKD-TML

immunization may prove to significantly increase the number of POL-specific B-cell precursors, antibody to flagella does not appear to play a significant role in protection since other studies have suggested that anti-flagella serum is not protective against lethal infections (see Roantree, 1967; Hohman et al., 1978). Moreover, Valtonen et al. (1977) found that S. typhimurium strains which were fla$^-$, i.e. strains lacking flagella, were as virulent as those strains which were fla$^+$. Taken together, these studies suggest that flagella do not significantly influence virulence. Therefore, it is possible that the flagella may be a good antigen, but not a protective one, or that the AKD processing of TML may alter the surface of the bacterium so that the antibody response is skewed more to the flagella-reactive B-cell precursors. However, recently, Carsiotis et al. (1983) derived a non-motile, non-flagellated S. typhimurium strain by transduction which was much less virulent than its motile isogenic partner. Their studies suggest that flagella may be important for virulence. Unlike Valtonen et al. (1977), these investigators studied the effects of the fla gene in Ity^s mice. Further, they did elaborate comparative growth kinetic studies in the RES tissues of both Ity^s and Ity^r mice (Weinstein et al., 1984). In addition, L.D.$_{50}$ values were determined for a length of 28 days, as opposed to only 10 days in the former study. Finally, preliminary results from M. Fultz and E.S. Metcalf (unpublished observation)

suggest that the antibody response to the non-flagellated S. typhimurium mutant which was derived by Carsiotis et al. (1983) is significantly lower than its flagellated isogenic partner. Thus, the stimulation of a strong anti-flagella antibody response with AKD vaccines may not be artificial.

Although few primary porin-specific B-cell clones were observed, the low frequency of B cells reactive with porin does not necessarily indicate that these cells play only a minor role in resistance to salmonella. As was the case with the memory POL-specific response, too few memory clones were analyzed for reactivity with porin. It is likely that examination of many more memory clones will also indicate a significant expansion of porin-specific clones after immunization. It is important to note, however, that S. typhimurium contains three species of porin in its outer membrane, each encoded by separate genes (Tokunaga et al., 1979a). These porin species from S. typhimurium are identified as 36K, 35K, and 34K, according to their molecular weights. Each species exists naturally as a trimer complex which acts as a diffusion pore for small hydrophilic compounds (Tokunaga et al., 1979b; Nakae, 1976). The porin preparation used in the studies described herein is composed only of 34K protein trimers. Therefore, it is possible that more porin-specific precursors may have been detected on immunoadsorbents made from one of the other two porin species or from all three species. To

date, it is not known if the immunogenicity of the three porins differs or what the degree of antigenic cross reactivity is between these three proteins. Tokunaga et al. (1979a) showed that the amino acid compositions of these species were very similar, but that differences did exist. Further, all three porin species had considerable differences in their tryptic peptide maps. However, Valtonen et al. (1977) have shown that recombinant strains of S. typhimurium which contain only one of the three porin species were as virulent as the parental S. typhimurium strain. Thus, none of the individual porin species appears to be a critical virulence factor. Moreover, porin preparations which contained only the 36K and 34K species were able to actively and passively protect mice against salmonellosis (Kuusi et al., 1979). This same porin preparation could also provide equivalent active protection against challenges with a S. typhimurium recombinant strain that expressed predominantly the 35K species and one that expressed predominantly the 36K species. These results suggest, indirectly, that each species of porin can cross-protect against the other species and that the overall porin-specific B-cell precursor frequency may not be very different from the 34K porin-specific B-cell frequency detected herein. However, this may not necessarily be true since the number of common and unique antigenic determinants within each porin species is unknown. In addition, minor clonotype differences are expected to

exist.

It is possible that infecting salmonellae are processed by the host's immune system in such a way as to expose antigens (both external and internal) which normally exist within obscure sites in intact organisms. Since the innermost moiety of the LPS molecule, lipid A, is an integral component of the outer membrane, it seemed possible that the bacterium could be processed such that new antigenic sites would be uncovered. These sites would not necessarily cross-react with determinants of the intact, free LPS molecule. Indeed, the results show that a very small proportion of primary CBA/Ca TML-specific B cells, which do not react with intact LPS, is directed to a lipid A antigenic determinant. Although the response is not, by any means, overwhelming, it does support the hypothesis that obscure antigenic sites are uncovered by a host processing mechanism. Although these particular lipid A determinants do not appear to be important immunogens, it is possible that other hidden or internal antigens may turn out to play a prominent role in the B-cell response.

Very little is known about the topology of the gram-negative bacterial surface in terms of the physical relationship between different surface structures. Interestingly, approximately 18% of the total primary TML-specific B-cell clones produce antibodies which react with more than one outer membrane component. Results presented

herein imply that these structures, specifically the flagella, porins, and lipid A, have the capacity to twist or fold about each other. Thus, the point of interaction between these structures produces a "combinational" antigenic determinant. Antibodies that react with these determinants have the capacity to partially bind to each of the purified antigens which compose the combinational antigenic site. It is important to note that only LPS-negative anti-TML antibody-producing clones were tested for reactivity against these three other antigens. Although the POL and porin preparations contain only trace amounts of LPS, it is possible that these levels could lead to false-positive results for LPS-positive clones being analyzed for reactivity against POL and porin. Ostensibly, LPS may also form "combinational" determinants with other surface structures. In fact, flagella and porins may not by themselves be potent immunogens but, in combination with LPS, may form major antigenic determinants which stimulate a significant proportion of the TML-specific B-cell repertoire. Therefore, many of the LPS-specific clones may also react with POL or the porin protein. Such a hypothesis is supported by the findings of Kuusi et al. (1981). These investigators discovered that their porin vaccine, which was able to both actively and passively protect mice from lethal salmonella challenge, contained substantial amounts of the Rb2 type LPS. If the LPS was removed, the protective capacity was lost in passive

immunization experiments. However, a large fraction of the anti-LPS antibodies could be removed from the protective rabbit antiserum with an LPS immunoadsorbent without loss of protection. They did not include a control to show that the same LPS immunoabsorption procedure could remove the protective capacity of an anti-LPS serum. However, the anti-LPS antibody activity was directed against the Rb2 type LPS, thereby eliminating the possibility that protection was due to anti-O-antigen antibodies. Their data suggested that it was the presence of complexes of porins and LPS which was essential for the generation of a protective immune response. Therefore, in order to determine if this hypothesis is correct, LPS positive anti-TML antibody-producing clones must be analyzed against POL and porin preparations which have been passed over an anti-LPS immunoadsorbent column.

B. Analysis of the Peyer's Patches TML-Specific B-Cell Repertoire in CBA/Ca Mice

Soon after oral challenge with virulent salmonellae, the infecting bacteria begin to multiply within the caecum and large intestine. Increasing numbers of viable salmonellae can be recovered primarily from the duodenal and ileal Peyer's patches, subsequently from the mesenteric lymph nodes, and eventually from the liver and spleen; henceforth, a systemic infection will be established (Collins and Carter, 1978; Carter, 1975; Carter

and Collins, 1974). Although the primary focus in the natural infection pathway of murine typhoid is the Peyer's patches, splenic involvement always follows even after sublethal challenges (Carter and Collins, 1974). Consequently, it is not surprising that Peyer's patches and spleen have equivalent numbers of TML-specific B cells (Table 21). However, Peyer's patches are continually exposed to normal gut flora, such as E. coli, which contain cross-reactive antigenic determinants. These intestinal bacteria may chronically stimulate and, thus, expand S. typhimurium reactive clones similar to the expansion described in chronically PR8 infected mice (Thompson et al., 1983). This type of clonal expansion would conceivably yield an increase in TML-specific B cell precursors. But, such cross-reactive exposure may also tolerize these B cells in much the same way as was hypothesized for splenic TML-specific precursors (see section A). Therefore, both spleen and Peyer's patches could have similar TML-specific B-cell frequencies. These results are consistent with the responses of splenic and Peyer's patches B cells to other antigens (Gearhart and Cebra, 1979; Fuhrman and Cebra, 1981; Cebra et al., 1982).

Although Gearhart and Cebra (1979) found that the overall frequency of PC-specific B cells was similar in Peyer's patches and in the spleen, the fine specificity of these two B-cell subsets differed. The majority of splenic

B cells were found to express the TEPC 15 (T15) idiotype whereas the majority of cells from Peyer's patches expressed non-T15 idiotypes. Previous studies by Sigal (1976) analyzed the idiotype specificity of splenic B-cell precursors responsive to PC in individual conventional and germ-free mice. His results showed that, although the frequency of $T15^+$ B-cell clones was not significantly different between these two donor pools, non-T15 precursors could represent up to 75% of the response in individual conventional donors. In contrast, germ-free mice never had more than 20% and, in many cases, had no non-T15 clones. Taken together, the studies of Gearhart and Cebra and Sigal suggest that environmental antigen exposure may selectively stimulate certain clones, such as $T15^-$ idiotypes in anti-PC responses. Thus, splenic PC-specific B cells in conventional mice will be influenced by normal gut flora to a much greater degree than those B cells in germ-free mice. Hence, spleens from conventional mice may have a larger proportion of $T15^-$ PC-specific B cells. Similarly, Peyer's patches B cells will be influenced even more than splenic B cells by environmental antigens since they are in continuous contact with normal gut flora. Additional studies by Gearhart and Cebra (1979) support this conclusion. They immunized mice by stomach intubation with the embryonated eggs of the PC-containing nematode, Ascaris suum. The frequency of PC-specific B cells increased in both the spleen and Peyer's patches with selective

expansion of $T15^-$ clones. Therefore, prior contact with an intestinal organism can preferentially stimulate non-T15 PC-specific precursors in either lymphoid organ. Their studies suggest that if the spleen was in contact with normal gut flora to the same extent as the Peyer's patches, then the number of non-T15 PC-specific precursors in the spleen would be equivalent to the number in the Peyer's patches. Ostensibly, different levels of cross-reactive antigen exposure account for differences in the clonal composition of the B-cell repertoire between the spleen and Peyer's patches.

Because the Peyer's patches may be exposed to some normal flora which may not spread to the spleen, different clonotypes could be preserved and/or deleted from the Peyer's patches B-cell pool by the T-cell suppression mechanism proposed by Thompson et al. (1983). Therefore, particular clonotypes selected in Peyer's patches would reflect the composition of the primary B-cell pool at the time of antigen exposure. Since the spleen may not be exposed to all the gut organisms that Peyer's patches are exposed to, the same clonotypes which are preserved in Peyer's patches may not be expressed in the spleen. On the other hand, clonotypes which may be deleted from the Peyer's patches B-cell repertoire may be expressed in the spleen. Moreover, encounter with cross-reactive environmental antigens in Peyer's patches may have

tolerized immature B cells in neonatal mice (Metcalf and Klinman, 1976). These tolerized clonotypes may be present in the spleen if these particular antigens do not spread to the spleen. These differences in the clonal composition would not necessarily create differences in precursor cell frequencies.

Too few clones derived from TML-specific B cells within the Peyer's patches have been examined to conclusively support this hypothesis. Hence, with caution, it is noted that there are no clones from the Peyer's patches which are reactive with the LPS molecule. In contrast, over 30% of those clones derived from splenic B cells in the same experiment are LPS-specific. The results of the analysis of this small group of splenic B-cell clones are consistent with the mean TML-specific primary B-cell response. Thus, these results may indicate real differences in fine specificity between Peyer's patches and splenic B cells with respect to LPS reactivity. Further, distinct clonotype expression between these two lymphoid tissues may exist in the group of clones which does not react with any of the defined determinants tested. It is also possible that the AKD-TML antigen preparation does not adequately distinguish the clonal composition within these tissues. This antigen preparation may stimulate particular clonotypes which may not normally be expressed in either tissue. Conversely, some clonotypes which are normally expressed in response to the viable organism may not be

stimulated by AKD-TML.

Since Peyer's patches contain cell populations enriched in precursors for IgA plasma cells (Craig and Cebra, 1974; Jones et al., 1974; Cebra et al., 1977), the isotype profile observed in the small study of Peyer's patches TML-specific clones is unexpected. Only a small proportion of the response, which is predominantly of the IgG2 isotype, expresses IgA. However, the isotype profiles of the splenic B cells analyzed is not completely typical either. Therefore, it is possible that this analysis of the Peyer's patches B cells is not truly representative of the Peyer's patches B-cell repertoire. Gearhart and Cebra (1979) found that, indeed, IgA was the predominant isotype expressed by Peyer's patches B cells which had been stimulated by either of two bacterial determinants, PC or inulin. Conversely, Fuhrman and Cebra (1981) found that, similar to the TML-specific precursors, only a small proportion of cholera toxoid-specific clones derived from the Peyer's patches secreted IgA. However, these results are based on the examination of only 20 clones. Analysis of additional cholera toxoid- and TML-specific clones may reveal that the response to cholera toxoid by Peyer's patches B cells is actually similar, in terms of isotype expression, to the responses of PC and inulin.

C. Analysis of the TML-Specific B-Cell Repertoire in CBA/N Mice

Previous studies have shown that the xid gene expressed by CBA/N mice and F_1 males derived from CBA/N females confers susceptibility to S. typhimurium (O'Brien et al., 1979) and a B-cell defect (Scher, 1982). Furthermore, results from this thesis and O'Brien et al. (1981) have shown that, although delayed in time of appearance and diminished in titer, anti-TML antibody can be detected in the serum of CBA/N mice after immunization with AKD-TML. However, primary TML-specific precursors are not detectable in either the spleen or Peyer's patches of these salmonella-susceptible, immune-defective mice. Interestingly, Eldridge et al. (1983) showed that CBA/N and F_1 male Peyer's patches cell cultures could support in vitro PFC responses to the TD antigen, SRBC, and to the TI-2 antigen, TNP-FIC. The results showed that spleen cell cultures from these immune-defective mice were unresponsive to these two antigens and it was proposed that unresponsiveness was due to the lack of mature Lyb-5^+ B cells. However, Peyer's patches from immune-defective mice were shown to contain a B-cell subpopulation with mature characteristics, putatively the Lyb-5^+ subset. These investigators contend that in the Peyer's patches, cells regulated by the xid gene overcome the block in maturation because of chronic stimulation with normal gut flora. If this is indeed the case, one would certainly expect to find

cells responsive to a bacterium like salmonella since this organism would probably be more cross-reactive with environmentally-induced antibodies than would TNP. It is possible that stimulation of TML-specific Peyer's patches B cells may have different activation requirements than splenic B cells. This seems unlikely since responses for the CBA/Ca controls do not appear suboptimal. In addition, the AKD preparation may not effectively stimulate the Lyb-5^+ precursors in CBA/N mice. Nonetheless, this preparation is able to stimulate precursors in CBA/Ca Peyer's patches. Thus, this antigen is effective in detecting differences between the CBA/Ca and CBA/N B-cell repertoires.

Primary PC-specific B-cell precursors from immune-defective spleens also fail to respond in the splenic focus system (Clough et al., 1981; Kenny et al., 1983). Until recently, all studies indicated that immune-defective mice could not express a functional PC-specific repertoire (Mond et al., 1977; Quintans, 1977). However, Kishimoto et al. (1979) and Clough et al. (1981) have shown that these mice can express a primary IgE anti-PC serum antibody response to low doses of antigen. Antibodies of other isotypes could not be detected. But, when high doses of antigen in CFA were administered to immune-defective mice (Wicker et al., 1982; Kenny et al., 1983), serum IgG anti-PC antibody was produced late in the primary response as well as in the secondary response. However, they failed to produce

significant amounts of IgM in either response. The antibodies produced by these mice in both the primary and secondary responses were predominantly T-15$^-$. Furthermore, recent studies (Clough et al., 1981; Kenny et al., 1983) have been able to detect secondary precursors in immune-defective mice which were responsive to PC-Hy in the splenic focus system. Therefore, mice which express the xid gene do contain at least some PC-specific B cells. Ostensibly, PC-responsive cells should be detectable in CBA/N Peyer's patches, particularly if this tissue contains Lyb-5$^+$ B cells. However, immune-defective Peyer's patches cells fail to produce anti-PC PFC responses to PC-Hy in an in vivo adoptive transfer system[1] (J.J. Kenny, personal communication). It is not yet known if immune-defective Peyer's patches cell cultures can support an in vitro PFC response to PC-Hy. Nevertheless, the lack of responsiveness of immune defective Peyer's patches B cells to both TML and PC suggests that it is not the alteration of the antigen by the AKD inactivation process which prevents adequate stimulation. It may be that environmental TML-cross-reactive and PC-containing

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[1]This lack of response has been observed in three separate experiments. Most recently, however, Peyer's patches cells from immune-defective mice, which had been obtained from a different animal facility, responded to PC-Hy in this system. Furthermore, the anti-PC PFC responses by these cells were much greater than the responses generated by normal controls. The reasons for this discrepancy in responsiveness of immune defective Peyer's patches B cells is unclear at this time.

antigens, rather than chronically stimulating Lyb-5$^-$ B cells to mature into Lyb-5$^+$ B cells, as proposed by Eldridge *et al.* (1983), tolerize TML-specific and PC-specific precursors so that Lyb-5$^+$ precursors responsive to these antigens do not develop.

CBA/N mice do not express primary TML-specific precursors nor do they express secondary precursors after priming with AKD-TML. However, after three immunizations, the CBA/N tertiary frequency is detectable. Although this frequency is similar in magnitude to that of primary CBA/Ca TML-specific B cells, the isotype profile and fine specificity of the two responses are characteristically different. Further, the tertiary CBA/N B cells appear to be memory cell-like in nature since: 1) very little IgM antibody is produced; 2) the proportion of IgG1 and IgG2 antibodies is much higher than in the primary CBA/Ca response; and 3) the majority of the anti-TML antibody is LPS-specific. Yet, these cells are still quantitatively and qualitatively different from CBA/Ca memory precursor cells immunized with the same regimen. Although the majority of the memory CBA/Ca anti-TML response is LPS-specific, almost half of the B cells are directed against the KDO or lipid A moiety. In contrast, only a few tertiary CBA/N clones are specific for this portion of the LPS molecule, the majority are reactive against the O-antigen region only. In addition none of the CBA/N clones were reactive against *S. pneumoniae*. These results suggest

were reactive against S. pneumoniae. These results suggest that KDO/lipid A-specific and S. pneumoniae cross-reactive precursors are restricted to the Lyb-5^+ B-cell subset. This restriction is compatible with the idea that lipid A could tolerize Lyb-5^- B cells since the lipid A region is structurally similar in most gram-negative bacterial organisms (Lüderitz et al., 1971) and because LPS is released into the surrounding environment (Collins, 1974). Tolerance may also account for the restriction of other responses in the Lyb-5^+ B-cell subset. For example, Wicker et al. (1982) found that the IgM T15$^+$ anti-PC antibodies of normal mice are derived from Lyb-5^+ B cells and that IgG T15$^-$ anti-PC antibodies are derived from Lyb-5^- B cells. Further, Stein et al. (1983) demonstrated that the Lyb-5^+ B-cell subset is responsible for the antibody response to an antigenic determinant in bacterial $\alpha(1\rightarrow6)$-linked dextran.

Kenny et al. (1983) offered a hypothesis which would explain: 1) the inability to activate primary Lyb-5^- B-cell precursors responsive to PC-Hy in splenic focus cultures; 2) the ability to generate a delayed in vivo primary IgG anti-PC response but not an IgM response; and 3) the ability to activate in vitro secondary anti-PC B-cell precursors. Their results suggested that PC-specific precursors in immune-defective mice must differentiate into memory cells before they can be activated to secrete antibody. They envisaged different activation requirements

for Lyb-5$^-$ and Lyb-5$^+$ PC-specific B cells. Consequently, they presumed that the splenic focus system must not provide all the necessary activation signals to primary Lyb-5$^-$ PC-specific B cells. The results presented in this thesis for TML-specific responses in immune-defective mice are remarkably similar to those of PC-specific responses in these mice. Thus, in accordance with the hypothesis made by Kenny et al. (1983), it can be postulated that Lyb-5$^-$ TML-specific (or PC-specific) B cells, upon primary stimulation, can not secrete antibody because they are unable to receive helper cell signal(s) that induces maturation to immunoglobulin secretion. However, these precursors can receive the helper signal(s) that activate(s) memory B cell formation. After repeated immunizations, class switching events proceed and these Lyb-5$^-$ B cells acquire a receptor for helper cell maturation signal(s). Therefore, secretion of anti-TML (or anti-PC) antibody can begin. As opposed to the Lyb-5$^-$ B-cell subset, unprimed Lyb-5$^+$ B cells, being a more mature subset, are able to secrete antibody after only a single immunization. Since they express receptors for both the proliferation signal(s) and maturation signal(s), Lyb-5$^+$ B cells can secrete antibody before class switching events have occurred. Thus, IgM anti-TML (or anti-PC) antibody is likely to be found in responses of Lyb-5$^+$ B cells but not in those of Lyb-5$^-$ B cells. This is supported by the data presented in Table 27.

Recent studies (Boswell et al., 1980a,b; Singer et al., 1982; Hodes et al., 1982) using Mishell-Dutton dissociated spleen cell cultures have shown that activation of Lyb-5^- and Lyb-5^+ B cells require very different cellular interactions even when responding to the same antigen and when activated by the same cloned population of T helper cells. Further, Mongini et al. (1982, 1983) have provided evidence which suggests that T cells can supply additional proliferative signals which enhance switching events within expanding B-cell clones. Moreover, the results herein suggest that although immune-defective TML-specific B-cell precursors may possess a receptor for the T helper signal(s) that activates differentiation, isotype switching events within these cells are mediated by a pathway(s) distinct from that of normal TML-specific B cells. It appears that after stimulation of CBA/Ca mice by AKD-TML, proliferation of an IgM-positive precursor cell ensues, followed by varying degrees of isotype switching within separate daughter cells of the B-cell clone. Whether these switches from IgM to other isotypes occur as sequential events within one lineage of daughter cells or as distinct events within separate daughter cells is not clear. However, these data suggest that, unlike TNP-FIC (Mongini et al., 1982, 1983), AKD-TML induces distinct switching pathways since there is no apparent correlation between the frequency of clones secreting each isotype and

the 5' to 3' IgCH gene order. There has been evidence that both sequential and distinct switching events can occur within clones of cells derived from single PC-specific B cells (Gearhart et al., 1980). Nevertheless, by whatever mechanism, AKD-TML stimulated normal B cells can undergo successive heavy chain class switches which yield expression of all five isotypes at some point in time. Upon repeated immunization, switches occur more frequently. In contrast, there appears to be only one switching pathway in immune defective mice. Although switches to the IgG2 isotype predominate in memory CBA/Ca B-cell clones, this isotype is generally secreted with other isotypes. The isotype pattern expressed by tertiary CBA/N clones indicates that a single distinct IgM-> IgG2 switching pathway exists for Lyb-5$^-$ TML-specific B cells. Occasional switches to other isotypes may occur to a minor extent which would account for the low levels of these isotypes observed. This implies that Lyb-5$^-$ B cells lack a receptor(s) for helper switch signals which mediate the expression of other isotypes. Although not examined in these studies, results of past studies (Kishimoto et al., 1979; Clough et al., 1981) suggest that a distinct IgE switching pathway may also exist in immune-defective mice since these mice produced significant levels of primary IgE anti-PC serum antibody and only poor levels of IgM, IgG, and IgA anti-PC antibodies. The likelihood that independent, IgG, IgA, and IgE switch pathways can occur

has been substantiated by several independent lines of evidence (Gearhart et al. 1980; Kuritani and Cooper, 1982; Kishimoto and Ishizaka, 1973; Kimoto et al., 1977; Mongini et al., 1982, 1983).

Collectively, these data suggest several factors which may be involved in the xid-conferred susceptibility of CBA/N mice to S. typhimurium infection. The original premise was that CBA/N mice may contain a reduced number of salmonella-specific B-cell precursors. Through the utilization of the splenic focus system, the determination of the frequency of such cells was attempted. At the present time, it can not be definitely stated that these mice do have reduced numbers of TML-specific B cells. Indeed, these studies suggest that they do not have primary or secondary precursors. In addition, the frequency of tertiary immune-defective precursors is 23-fold lower than tertiary precursors in normal CBA/Ca mice. However, Clough et al. (1981) has shown that after repeated immunization the frequency of PC-specific precursors in immune-defective mice was nearly equivalent to that in normal mice. The similarities between the responses to TML and PC-Hy by these mice is striking. Therefore, it is possible that after more immunizations, the frequency of TML-specific precursors in immune-defective mice will also begin to approach the frequency observed in normal mice. If, after several rounds of AKD-TML challenge, the frequency within

these mice remains low, then the original premise would be definitely confirmed. Whether or not the frequency of CBA/N-derived TML-specific precursors increases after hyperimmunization, the delayed development of these cells may play a very important factor in the susceptibility of these mice to infection. Kenny et al. (1983) proposed that Lyb-5^+ PC-specific precursors are responsible for the primary in vivo response and that the Lyb-5^- subset only plays a role in the secondary in vivo anti-PC response. Again, the results of the TML-specific response in CBA/N mice are compatible with this hypothesis. However, it has not been shown, if, in fact, primary Lyb-5^- B cells from normal CBA/Ca mice can respond to AKD-TML in the splenic focus system. It is possible that the Lyb-5^- B-cell subset may be composed of two populations: one, which has more stringent activation requirements, is present in both CBA/Ca and CBA/N mice, and one, which has similar activation requirements as Lyb-5^+ B cells, is present only in CBA/Ca mice. Other studies have provided evidence which supports the existence of two Lyb-5^- subsets (Metcalf et al., 1980; Kung et al., 1982; Ono et al., 1983). Metcalf et al. (1980) investigated the susceptibility of immune defective splenic B cells to tolerance induction. Although essentially all of these B cells are Lyb-5^-, approximately 50% of these cells were not tolerizable. In addition, Kung et al. (1982) described a rat monoclonal antibody which stained only 30% of normal B cells and 60% of immune-

defective B cells. Approximately 50% of normal B cells are Lyb-5$^+$ (Ahmed et al. 1977; Ahmed and Singer, 1979). Therefore, this monoclonal antibody appears to be specific for a subpopulation of Lyb-5$^-$ B cells. Finally, data presented by Ono et al. (1983) demonstrated that normal neonatal and adult Lyb-5$^-$ B cells were able to generate an anti-TNP PFC response to TNP-LPS(phenol) whereas immune-defective B cells were not. These results support the hypothesis that Lyb-5$^-$ B cells are composed of two subsets. Ono et al. proposed that these two B-cell subsets represent different developmental stages. The subset present in immune-defective mice may represent the most immature stage while the other Lyb-5$^-$ subset, which these mice lack, may represent a developmental stage intermediate between the most immature Lyb-5$^-$ B cells and the mature Lyb-5$^+$ B cells. Instead of two distinct Lyb-5$^-$ subsets, the xid gene may also exert its control on the activation of Lyb-5$^-$ B cells. Thus, primary Lyb-5$^-$ TML-specific B cells from normal mice would be detectable. However, this is unlikely since primary PC-specific Lyb-5$^-$ B cells from normal mice are undetectable (Kenny et al., 1983). Although Lyb-5$^-$ B cells may be involved only in secondary responses, by themselves, these B cells are apparently ineffective in providing resistance to S. typhimurium infection. Ostensibly, if secondary Lyb-5$^+$ TML-specific B cells are more easily activated, this subset would be able to keep

the infecting salmonellae in check until anti-TML antibody production occurs by the Lyb-5$^-$ B cell subset. In immune-defective mice the development of these antibodies may be too late. In addition, without anti-TML antibodies produced by Lyb-5$^+$ B cells, those derived from Lyb-5$^-$ B cells may be ineffective in handling the growing infection.

Although hyperimmunization may increase the frequency of TML-specific precursors in CBA/N mice, these data indicate that the anti-TML repertoire in immune-defective mice may be lacking a very important subset of TML-specific B cells. In spite of the fact that the majority of anti-TML antibodies derived from CBA/N mice are LPS-specific, only a small proportion are KDO/lipid A-specific. Because almost half of the LPS-specific anti-TML antibodies from normal, memory CBA/Ca clones are directed to KDO/lipid A, this deficiency in immune-defective mice may be deleterious. CBA/N mice also appear to be lacking another subset of TML-specific B cells, although this subset and the KDO/lipid A-specific subset may not be mutually exclusive. This missing subset of B-cell precursors has the capacity to switch to isotypes other than IgG2. The comparison of the CBA/Ca and CBA/N repertoires demonstrates that IgG2 anti-TML antibody is predominant in the normal primary and memory responses and that Lyb-5$^-$ B cells are presumably responsible for the increase in IgG2 production in memory responses. However, it appears that those B cells whose clonal progeny can

express other isotypes by class switching events are also important in the memory response of normal resistant mice. It is important to note that it has not been determined what proportion of the anti-TML response is IgG2b and what proportion is IgG2a. It is possible that distinct switching pathways exist for these two subclasses and only one of these may be expressed in CBA/N mice.

Though CBA/N mice are apparently lacking IgG3 anti-TML B-cell precursors (Table 27), this deficiency may not be so deleterious in terms of their immune response to S. typhimurium since IgG3 anti-TML antibody comprises less than a quarter of the memory response in normal CBA/Ca mice. On the other hand, molecular studies by Shimizu et al. (1982) have shown that the switch region 5' to the Cu gene shares more homologous sequences with the IgG3 switch region than with any other IgG region. In addition, the IgG3 switch region has more homology with other IgG regions than does the IgM switch region (Lang et al., 1982; Stanton and Marcu, 1982). Therefore, although secretion of IgG3 anti-TML antibody may not be important or may only be transiently produced in the immune response to S. typhimurium, those IgG3 B cell precursors missing in CBA/N mice (probably Lyb-5^+ B cells) may express the CH gene which contains the switch region that allows the expression of all the isotypes by clonal progeny. Recently, Mongini et al. (1982, 1983) demonstrated that the majority of TNP-

FIC stimulated clones which secreted IgG1, IgG2b, and IgG2a also secreted IgG3. Their data are consistent with this hypothesis since TNP-FIC appears to stimulate only Lyb-5^+ B cells (Ahmed et al. 1979; Boswell et al. 1980a).

Clearly, the TML-specific B-cell repertoire in CBA/N mice is different from that in CBA/Ca mice. Whether the differences outlined in this thesis determine the susceptibility of these animals to S. typhimurium still remains to be determined. Furthermore, additional distinctions have yet to be defined. For example, although the majority of anti-TML antibody-producing clones derived from CBA/N mice are directed against the O-antigen region of the LPS molecule, the carbohydrate specificity of these antibodies may differ from those O-specific antibodies derived from CBA/Ca clones. S. typhimurium strain TML expresses the O:1,4,12 antigens. The O:1 determinant is defined as a α-glucose 1,6-galactose disaccharide; the O:4 determinant as the dideoxyhexose, abequose, bound α1->3 to mannose; and the O:12 determinant as trisaccharide subunits of mannose, rhamnose, and galactose (Lüderitz et al., 1966; Svenson and Linberg, 1978; Jorbeck et al., 1979). Therefore, it is possible that the Lyb-5^- subset may be lacking those O-specific B cells which play a major role in the memory response. By analyzing both tertiary CBA/N and memory CBA/Ca O-specific monoclonal anti-TML antibodies in an RIA using a variety of Salmonella strains as

it should be possible to determine if immune-defective mice do indeed lack an O-specific B-cell subset which may, in turn, be necessary for protective immunity. As mentioned earlier, additional deficiencies in the CBA/N precursor pool may become apparent as clones are further analyzed on a more extensive panel of bacterial components such as 36K and 35K porin proteins, ompA protein, lipoprotein (DiRienzo et al., 1978) and the enterobacterial common antigen (Valtonen et al., 1976).

Often it is difficult to see the forest for the trees. By analogy, it is a difficult task to evaluate the S. typhimurium-specific B-cell repertoire because of the complexity of the organism. However, even with all the noted limitations of such an endeavor, the studies presented herein make some valuable contributions to the understanding of humoral immune mechanisms which may ultimately be involved in protection against an infectious disease agent. The AKD preparation as well as the conditions of the system used to analyze TML-specific precursors in CBA/Ca and CBA/N mice were able to stimulate a wide array of TML-specific B cells. Although the precursors which were stimulated may not represent the entire TML-specific B-cell pool, all three populations (primary CBA/Ca, memory CBA/Ca, and tertiary CBA/N B cells) were studied and compared under the same conditions. Under these conditions, the isotype profile and fine specificity

analysis of the TML-specific clones not only reflect differences among these three B-cell populations, but also indicate an abnormal anti-S. typhimurium B-cell response in CBA/N mice. In addition, the responsiveness to AKD-TML by both normal and immune-defective mice reported in this thesis is compatible to the responses to two other natural antigens, HA and PC. The precursor frequency specific for each one of these three antigens is very low in normal mice. Neither TML nor HA stimulate a prominent IgG1 response, but IgG2 and IgA appear to be important in responses to natural antigens. Further, neither PC nor TML can activate primary splenic or Peyer's patches immune-defective precursors in the splenic focus system. These latter two antigens, as well as bacterial $\alpha(1\rightarrow6)$ dextran appear to elicit antibodies of restricted isotypes that are clonally restricted to distinct B-cell subsets.

The remarkable similarities in B-cell responsiveness to different natural antigens support the validity of the findings presented in this thesis. Although much remains speculative at this point, it is hoped that these studies have provided a fundamental framework with which to further dissect and characterize the biologically relevant murine B-cell repertoire to S. typhimurium. As these studies progress many of the limitations should be resolved. The utilization of the viable organism and its components should provide further relevance to these studies. Finally, it is possible that

as the response is separated into its constituent parts, a portion of the response may in fact be typical of classical anti-synthetic antigen responses. Certainly, these studies should provide insights on the role of B cells in resistance to murine typhoid.

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